

In vitro cytotoxic, antioxidative and α -glucosidase inhibitory potential of a herbal mixture comprised of *Allium sativum* and *Lagerstroemia speciosa*

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Abstract. – **BACKGROUND,** Hyperglycemia induced over production of free radicals in the mitochondrial electron transport chain is now considered as one of the central mechanisms in the pathogenesis of diabetic complications. *Allium sativum* and *Lagerstroemia speciosa* contains active principles possessing anti-diabetic and antioxidant properties. This study is aimed at evaluating the evidence that supports this traditional claim and investigates the possible synergistic effect on these herbs when given as a herbal mixture *in vitro*.

AIM, The present study investigates the cytotoxic, antioxidant and α -glucosidase inhibitory potential of *Allium sativum* (ASE), *Lagerstroemia speciosa* (LSE) and their combinations using *in vitro* methods.

MATERIALS AND METHODS, The total phenol, total flavonoid and total tannin content were determined in ASE and LSE. The cytotoxic effects of ASE, LSE and their combination in the ratio of 1:2, 1:1 w/w were evaluated using 3T3 L1 preadipocyte cells. Effect of ASE, LSE and its mixture on intracellular reactive oxygen species (ROS) production were determined by 2', 7'-dichlorofluorescein diacetate (DCF DA) staining technique in 3T3-L1 adipocytes. The ability of the herbal extracts and their combination to scavenge super oxide radicals and to inhibit α -glucosidase enzyme (a carbohydrate metabolising enzyme) were measured using *in vitro* methods.

RESULTS, The total phenols and tannins were expressed as microgram (μ g) of gallic acid equivalents/mg of extract (GAE/mg), flavonoids as μ g of quercetin equivalents/mg of extract (QE/mg). LSE had significant higher total phenol (300.11 ± 1.99), flavonoid (53.12 ± 0.48) and tannin content (118.90 ± 0.15) compared to ASE which possessed total phenol (159.93 ± 0.87); flavonoid (9.37 ± 0.73) and

tannin content (80.5 ± 0.19). The IC_{50} value, the concentration of the extracts that cause 50% inhibition or cell death was measured as an index of cytotoxicity. The IC_{50} value was found to be in the following decreasing order: 1:2 mixture (98μ g/ml) > ASE (323.6μ g/ml) > 1:1 mixture (428.1μ g/ml) > LSE (2154μ g/ml). The 1:1 mixture was comparatively less cytotoxic under the tested concentration range ($1 \times 10^0 \mu$ g – $1 \times 10^8 \mu$ g) than 1:2 combinations. The results observed with lactate dehydrogenase (LDH) release were similar to that of cell viability assay. The 1:1 mixture (DIA-2 hereafter) was considered for further investigations. DIA-2 inhibited the ROS levels, which is evidenced by the decreased DCF fluorescence. DIA-2 could also efficiently scavenge the super oxide radical generated from PMS/NADH-NBT system showing an IC_{50} value 69.99μ g/ml, the IC_{50} value of ASE (157.7μ g/ml), LSE (20.43μ g/ml), and ascorbic acid (49.64μ g/ml) used as positive control. The results of *in vitro* α -glucosidase inhibitory assay showed highest IC_{50} value with LSE (0.3μ g/ml) and DIA-2 (0.7μ g/ml) than ASE (136.3μ g/ml) and positive control miglitol (651.8μ g/ml).

CONCLUSIONS, DIA-2 exerts synergistic effect in scavenging the ROS and inhibiting the enzyme α -glucosidase *in vitro* compared to its individual extracts. The possible synergistic therapeutic effects may be due the presence of the antioxidant rich flavonoids, phenols and tannins present in LSE and ASE.

Key Words:

Allium sativum, *Lagerstroemia speciosa*, Garlic, Bana-ba, Super oxide radical scavenging, 3T3-L1 adipocytes, cytotoxicity, MTT assay, LDH release assay, α -glucosidase inhibitor, 2',7'-dichlorofluorescein diacetate.

Abbreviations

LSE = Lagerstroemia speciosa
ASE, Allium sativum
AA = Ascorbic acid
ROS = Reactive Oxygen Species
DCF-DA = 2, 7 Dichlorofluorescein diacetate
IC₅₀ = half maximal inhibitory concentration
PMS = Phenazine Metho Sulphate
NADH = Nicotinamide Adenine Dinucleotide (reduced form)
NBT = Nitro Blue Tetrazolium
DMEM = Dulbecco's Modified Eagle Medium
LDH = Lactate Dehydrogenase
AGE = Advanced Glycation End products
MTT = 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
GOD-POD = glucose oxidase-peroxidase
H₂O₂ = hydrogen peroxide
GAE = gallic acid equivalent
QE = Quercetin equivalent
FCR = Folin-Ciocalteu's Reagent

Introduction

Hyperglycaemia is the major cause in the development of micro and macrovascular diabetic complications¹. Hyperglycemia induced mitochondrial overproduction of reactive oxygen species (ROS) activates the major pathways involved in the pathogenesis of complications namely, polyol pathway flux, increased formation of advanced glycation end products (AGE), increased expression of the receptor for AGEs, activation of protein kinase C isoforms, and overactivity of the hexosamine pathway². Pharmacologic inhibition of mitochondrial ROS overproduction in hyperglycaemic state could prevent glucose mediated vascular damage^{3,4}. This hypothesis offers a new concept for drug discovery⁵. The current drug developmental strategy has targeted on oxidative stress pathways for the prevention of diabetic vascular complications⁶. It is well known that single therapeutic drug could not prevent the multi-factorial pathogenesis of diabetes or manage the hyperglycemia induced oxidative stress. Synthetic agents that inhibit the various biochemical pathways involved in the pathogenesis of diabetic complications are under clinical trial and the results obtained so far are either disappointing or inconclusive⁷. Herbal medicines are been traditionally used for the treatment of diabetes and its

complications and the presence of multiple phytochemical compounds present in herbal medicines provide a promising multiple therapeutic approach for the prevention and treatment of diabetic complications⁶. *Allium sativum* and *Lagerstroemia speciosa* have a long history in folk medicine and are well known medicinal plants traditionally used for the treatment of various disorders including diabetes mellitus. DIA-2 is a unique combination of 1:1 w/w ratio of extracts from these two herbs and was prepared with the intention to combat hyperglycemia and oxidative stress induced diabetic complications. The unique features of this formulation are that it contains standardised extracts *Allium sativum* (1.1% alliin w/w) and *Lagerstroemia speciosa* (1.28% w/w corosolic acid). The minimum number of ingredients was used in the formulation compared to other commercial multi-herbal formulation. The present study aims to investigate the cytotoxic effect of *Allium sativum* and *Lagerstroemia speciosa* and their combinations *in vitro* using 3T3-L1 adipocytes. The individual herbs and the composition showing comparatively low cytotoxicity (DIA-2) were further qualitatively investigated for their ability to inhibit the production ROS in preadipocytes using dichlorofluorescein diacetate (DCF-DA) staining assay. In addition, DIA-2 and its individual ingredients were investigated for *in vitro* super oxide radical scavenging and *in vitro* antihyperglycemic activity by evaluating their ability to inhibit carbohydrate digestive enzymes like α -glucosidase.

Materials and Methods

Chemicals and Reagents

Phenazine methosulphate (PMS), Nicotinamide adenine dinucleotide phosphate reduced (NADH), Nitro blue Tetrazolium (NBT), Ascorbic acid (AA), α -Glucosidase were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd, Mumbai, India. Quercetin and Gallic acid, (3-(4, 5 Dimethylthiazol 2- yl) 2, 5 diphenyltetrazolium bromide (MTT) and dichlorofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich (Sigma Aldrich Chemical Co, St Louis, MO, USA). Miglitol (MG) was a kind gift from M/s.Orchid Chemicals & Pharmaceuticals Limited, Chennai, Tamilnadu, India. Diagnostic kit for glucose and LDH determination was purchased from Merck Ltd, Mumbai, India. All cell

culture solutions and supplements were purchased from Life Technologies Inc. (Gaithersburg, MD, USA). Dulbecco's Modified Eagle Medium (DMEM) was obtained from GIBCO, BRL (Gaithersburg, MD, USA). All other reagents were of analytical grade.

Test Extracts

Aqueous extract of dried bulbs of *Allium sativum* (ASE) and 40% methanolic extract of dried leaves of *Lagerstroemia speciosa* (LSE) were obtained commercially from Amsar Pvt. Ltd, Indore, India and K. Patel Phyto Extractions Pvt Ltd, Mumbai, India respectively. Both ASE and LSE has been standardized and optimized at their respective commercial plants and claimed to contain 1.1% alliin w/w and 1.28% w/w corosolic acid respectively.

3T3-L1 Cell Line

The 3T3-L1 cell line, used in the present study, was obtained from National Centre for Cell Science (NCCS), Pune, India. Stock cultures of these cell lines were cultured as mentioned below and used for the study. Aseptic procedures were used in the handling of the cell cultures following standard operating procedures.

Phytochemical Evaluation of ASE and LSE

Preparation of Extract, Reagents and Standards

The extracts were prepared in 80% methanol. Folin-Ciocalteu's reagent (FCR) was prepared in the ratio of 1:9 and 1:2 in distilled water for phenolic content and tannin content estimation respectively. A stock solution (1 mg/ml) of standard gallic acid and quercetin were dissolved in methanol.

Total Phenol Content

Total phenol content was determined using the methods described earlier⁸. Briefly, to 1 ml of the extract [(ASE (100 µg/ml) or LSE (500 µg/ml) or gallic acid], 5 ml of FCR and 4 ml of sodium carbonate (7.5%) was added. The mixture was allowed to stand for 15 min at 37°C, after which absorbance was measured at 765 nm using spectrophotometer (Multiskan, Thermo Scientific, Rockford, IL, USA). A calibration curve was plotted using gallic acid (8-40 µg/ml) as standard. The amount of total phenols was expressed as gallic acid equivalent (GAE) in microgram per milligram of extract from a calibration curve.

Total Flavonoid Content

Total flavonoid content was determined using the methods reported earlier⁹. Briefly, 500 µl of the extract (1000 µg/ml) or quercetin was made up to 4.5 ml of methanol. 100 µl of aluminium chloride (10% w/v), 100 µl of sodium acetate (1 M) and 2.8 ml of distilled water were added. All the above reagents excluding the extract or quercetin served as blank. The tubes were incubated at room temperature for 30 min. The absorbance was measured at 415 nm (Multiskan, Thermo Scientific, Rockford, IL, USA). A calibration curve was constructed using quercetin (8-40 µg/ml). The total of amount flavonoids was expressed as quercetin equivalent (QE) in microgram per milligram of extract from a calibration curve.

Tannin Content

Tannin content was determined as per the method¹⁰ and the content was expressed as gallic acid equivalent (GAE) in microgram (µg) per milligram of extract from a calibration curve. Briefly, to 1 ml of the extract [ASE (200 µg/ml) or LSE (1000 µg/ml)], 1 ml of distilled water was added, followed by 0.5 ml of Folin's phenol reagent and 5 ml of sodium carbonate (35%) were added. After 5 minutes incubation at room temperature, absorbance was measured at 640 nm (Multiskan, Thermo Scientific, Rockford, IL, USA) against blank.

Cytotoxicity Assay of ASE and LSE and Their Different Combination Mixtures

Cell Culture and Maintenance

3T3-L1 preadipocytes were grown in T25 tissue culture flask containing Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (100 IU, 100 mg/ml, respectively) in a humidified atmosphere of 5% CO₂ at 37°C. Preconfluent 3T3-L1 cells were seeded in 96-well plates at a density of 8,000 cells/200 µl/well. Based on the percentage confluence, the cells were trypsinized and sub-cultured. During the process, the medium was replaced with fresh medium every 2-3 days. The cells were examined for morphology using an inverted microscope (Motic AE30, Hong Kong). The optimal cell concentration for 3T3 L1 adipocytes/well was optimised¹¹.

Measurement of Cell Viability

Cell viability experiment was carried out in 96 well micro titre plates as per the earlier described method¹². Optimal concentration of cells were treated with different concentrations (ranging from 1×10^0 μ g - 1×10^8 μ g) of individual extracts or combination after 24 h following plating and incubated at 37°C for one day. At 20 h following extract exposure, the cells were incubated in a humidified atmosphere of 5% CO₂ at 37°C with 5 mg/ml MTT for 4 h. At the end of the experiment, the medium was removed, and the insoluble formazan product was dissolved in dimethyl sulphoxide (DMSO) (200 μ l) and kept at least 15 min in the dark. MTT reduction was quantified by measuring the absorbance at 570 nm and 630 nm in spectrophotometer (Multiskan, Thermo Scientific, Rockford, IL, U.S.A). The percentage growth inhibition was calculated using the formula below:

$$\% \text{ cell growth inhibition} = 100 - \left\{ \frac{(At-Ab)}{(Ac-Ab)} \right\} \times 100$$

Where, At = Absorbance value of test, Ab = Absorbance value of blank, Ac = Absorbance value of control. Cytotoxicity was expressed as IC₅₀ value. The IC₅₀ value is the concentration of the extracts that cause 50% inhibition or cell death and was obtained by plotting the percentage inhibition versus concentration of the extracts.

Measurement of Cytotoxicity

For the LDH assay, the 3T3-L1 cells were plated in 96-microtiter plates. Twenty four hour following plating, optimal concentration of cells was exposed to varying concentrations of the DIA-2 and its individual ingredients. After 24 h of treatment, the cells were then treated with a cell lysis solution (DMSO) for 30 minutes at room temperature to lyse. Release of LDH in supernatant was assessed by using a LDH kit according to the procedures (Merck Ltd, Mumbai, India) mentioned in the kit insert. The intensity of the colour is proportional to LDH activity. The absorbance was determined at 490 nm with 96-well plate ELISA reader (Multiskan, Thermo Scientific, Rockford, IL, USA). The percent LDH release was calculated as follows: (LDH in culture supernatant/LDH in culture supernatant + LDH in cell lysate) \times 100.

Measurement of Intracellular ROS

Intracellular ROS scavenging activity was carried out using DCF-DA as described in earlier re-

ported methods¹³. Briefly, 3T3-L1 adipocyte cell was seeded on 48-well plate at 10,000 cells/well and allowed to attach overnight. Twenty four hours after plating, the cells were treated with varying concentrations (10 and 100 μ g/ml) of the extracts and incubated at 37°C. One hour later, 100 μ M of H₂O₂ was added to the plate. After 1 h, 10 μ M of DCF-DA solution was added and incubated for 1 h. The effect of extract on the extent of ROS production was visually observed and the cell images were captured using moticam digital camera fitted to inverted fluorescent microscope (Motic AE30, Hong Kong).

In vitro Super Oxide Radical Scavenging Assay

Super oxide radical scavenging assay was carried out as per the method described¹⁴. 0.05 ml of extracts and DIA-2 of concentrations ranging from 2-1000 μ g/ml were added to test tubes, followed by 0.3 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.025 ml of PMS (186 μ M) and 0.075 ml of NBT (300 μ M in buffer of pH 8.3) The reaction was initiated by addition of 0.075 ml of NADH (780 μ M in buffer of pH 8.3). After incubation at 30°C for 90 seconds, the reaction was stopped by addition of 0.25 ml glacial acetic acid. Then the reaction mixture was stirred vigorously and shaken with 2.0 ml of n-butanol. The mixture was allowed to stand for 10 minutes and centrifuged. 1.5 ml of n-butanol alone served as blank. Ascorbic acid served as the positive control. The colour intensity of the chromogen was read at 560 nm. Results were expressed as percentage of inhibition of superoxide radicals. The percentage scavenging activity was calculated using the formula: $[1 - (\text{Abs}_{\text{test}}/\text{Abs}_{\text{blank}})] \times 100$, where Abs_{test} – Optical density obtained in presence of test compounds and Abs_{blank} – Optical density obtained in absence of test compounds.

α -Glucosidase Inhibition Activity

Experiment was carried out by the method previously described¹⁵. Briefly, 200 μ l of α -glucosidase solution (0.6 U/ml) was pre-incubated with 200 μ l of various concentrations (1-1000 μ g/ml) of extract dissolved in ethanol: water mixture (8:2) or vehicle control for 5 min. The reaction was initiated by adding 200 μ l of the substrate (37 mM sucrose) and terminated after 30 min incubation at 37°C by heating at 90-100°C in a water bath. The formation of glucose

was determined by glucose-peroxidase (GOD-POD) method at 546nm using star-21plus biochemistry auto analyser (Rapid Diagnostic Pvt. Ltd, Delhi, India). The inhibitory effect of extracts on α -glucosidase was determined by the rate of decrease in the amount of glucose liberated from molecules of substrate after incubation with the enzyme. The IC_{50} values (concentration in $\mu\text{g/ml}$ required for 50% inhibition of α -glucosidase activity under the assay conditions specified.) were determined by non-linear regression analysis using GraphPad Prism (Version 4.03). The values are mean of triplicate incubations expressed as mean \pm SEM. Miglitol (1 to 1000 $\mu\text{g/ml}$) was used as positive control and α -glucosidase inhibition was carried out under the same assay conditions specified above.

Statistical Analysis

All the *in vitro* experimental results were expressed as mean \pm S.E.M of two parallel measurements. The concentration of the extracts or their combination that caused 50% of inhibition of the system assessed (IC_{50}) were determined by non-linear regression analysis using GraphPad Prism software (Version 4.03).

Results

Phytochemical Evaluation of ASE and LSE

The results of phytochemical analysis of ASE and LSE are shown in Table I. The amount of phenolic compounds present in ASE and LSE are 159.93 ± 0.87 and 300.11 ± 1.99 of GAE in $\mu\text{g/mg}$ of extract respectively. The flavonoid content available with ASE and LSE are 9.37 ± 0.73 and 53.12 ± 0.48 of QE in $\mu\text{g/mg}$ of extract respectively. The tannin content present in ASE and LSE are 80.5 ± 0.19 and 118.90 ± 0.15 of GAE in $\mu\text{g/mg}$ of extract respectively.

Table I. Total phenolic, flavonoid and tannins content of ASE and LSE.

Extract	Total phenol ¹	Total tannin ¹	Total flavonoid ²
ASE	159.93 ± 0.87	80.5 ± 0.19	9.37 ± 0.73
LSE	300.11 ± 1.99	118.90 ± 0.15	53.32 ± 0.48

¹Total phenols and ¹Total tannin expressed as GAE in $\mu\text{g/mg}$ of extract. ²Total flavonoid expressed as QE in $\mu\text{g/mg}$ of extract. Values were expressed as the Mean \pm S.E.M of two parallel experiments.

Cytotoxicity Assay of ASE and LSE and Their Different Combinations

The viability of 3T3-L1 preadipocytes accessed by MTT dye reduction assay is represented in Figure 1. The IC_{50} value (the concentration of the extract required to produce 50% reduction in cell viability) of ASE, LSE were 323.6 $\mu\text{g/ml}$ and 2154 $\mu\text{g/ml}$ respectively. ASE was found comparatively toxic than LSE. We attempted the cytotoxic effects of two possible mixtures of these extracts (1:2 and 1:1) keeping the ratio of ASE fixed as 1 part due to its cytotoxic nature. The IC_{50} value of 1:1 w/w mixture of ASE and LSE was found to comparatively low 428.1 $\mu\text{g/ml}$ than 1:2 mixtures (98 $\mu\text{g/ml}$). When compared to 1:2 mixtures, 1:1 w/w mixture suppressed the differentiation of preadipocytes to adipocytes without exerting significant cytotoxic effects (Data not shown). Based on these findings, 1:1 mixture (hereafter called as DIA-2) was considered for further investigations. The membrane protective effects of DIA-2 and its component herbs as determined by LDH release assay using 3T3-L1 preadipocytes are represented in Figure 2. At the test dose (1×10^8 μg) level the percentage of LDH release compared to the untreated vehicle control (22.16 ± 2.20) ASE (58.99 ± 5.90) and DIA-2 (55.60 ± 5.58) showed comparatively high LDH release into the culture medium than LSE (23.58 ± 2.36).

Effect of ASE, LSE and DIA-2 on Intracellular ROS Production

The ability of DIA-2 and its component herbs to mitigate oxidative stress was demonstrated by hydrogen peroxide (H_2O_2) induced oxidative stress in 3T3-L1 cells. The representative images obtained in the study are shown in Figure 3. After addition of 100 μM of H_2O_2 there was an increase in the production of ROS and increased number of fluorescent cells was observed in untreated cells. In 1 h pre-treatment of cells with

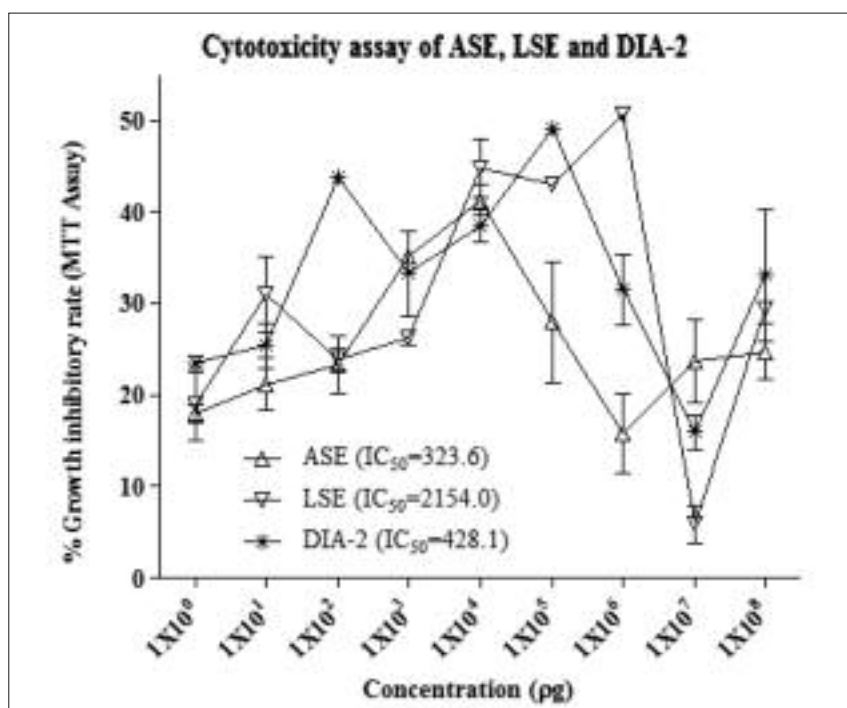


Figure 1. Cell viability analysed by MTT assay. Cytotoxicity results of ASE, LSE and 1:1 combination of ASE and LSE (DIA-2) on 3T3-L1 pre-adipocytes after 20 h exposure period. The values represent the mean \pm SEM (n = 2 wells) from a single experiment. The cytotoxicity results of 1:2 combination data are not shown.

100 μ g/ml of respective treatments (ASE, LSE, DIA-2 and ascorbic acid) there was distinct reduction in the generation of ROS as compared to untreated cells with DIA-2 and ascorbic acid treated cells.

Effect of ASE, LSE and DIA-2 Super Oxide Radicals Scavenging

The IC₅₀ values [the concentration (μ g/ml) of test compound that inhibits the formation of super oxide anion by 50%] obtained from super ox-

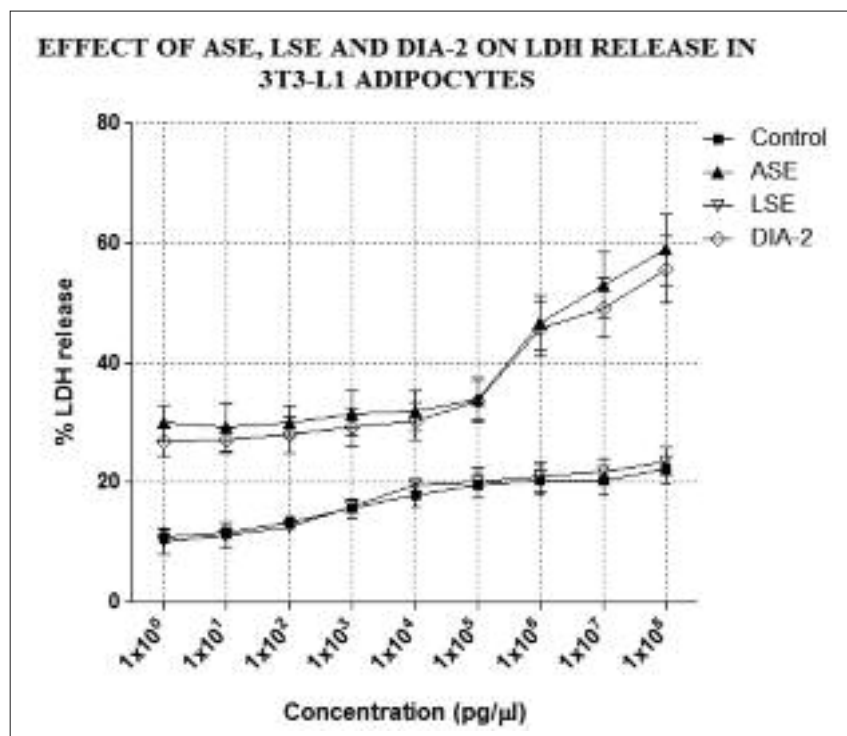


Figure 2. Cell viability analysed by LDH release assay. Effect of 20 h exposure to different concentrations of ASE, LSE and 1:1 combination of ASE and LSE (DIA-2) on 3T3-L1 pre-adipocytes. The values represent the mean \pm SEM (n = 2 wells) from a single experiment. The cytotoxicity results of 1:2 combination data are not shown.

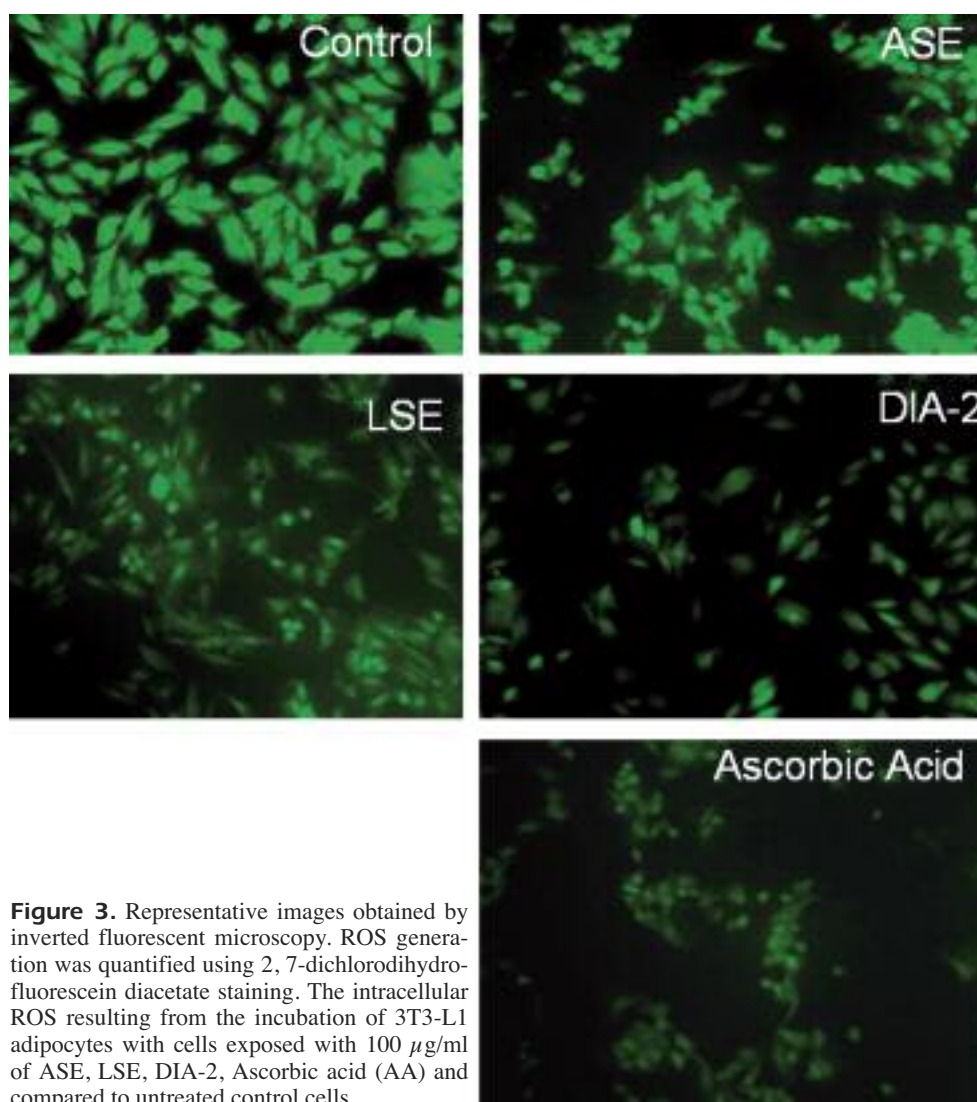


Figure 3. Representative images obtained by inverted fluorescent microscopy. ROS generation was quantified using 2, 7-dichlorodihydrofluorescein diacetate staining. The intracellular ROS resulting from the incubation of 3T3-L1 adipocytes with cells exposed with 100 $\mu\text{g/ml}$ of ASE, LSE, DIA-2, Ascorbic acid (AA) and compared to untreated control cells.

ide radical scavenging is depicted in Figure 4. The effect of extracts on scavenging the super oxide radicals generated from a non enzymatic (NBT–NADH–PMS) system decreased in the order of LSE (20.43 $\mu\text{g/ml}$) > Ascorbic acid (49.64 $\mu\text{g/ml}$) > DIA-2 (69.99 $\mu\text{g/ml}$) > ASE (157.7 $\mu\text{g/ml}$) under the tested experimental conditions. Super oxide anion reduces nitro blue tetrazolium (NBT) to formazan at pH 7.8 at room temperature. Formazan formed can be determined by measuring the optical density (OD) at 560 nm. The decrease in OD in the presence of extracts indicates the consumption of super oxide anion by the extracts.

Effect of ASE, LSE and DIA-2 α -glucosidase Inhibition

The effect of DIA-2 and its component herbs for their inhibitory activity against α -glucosidase *in vitro* compared to standard reference control miglitol is represented in Figure 5. The inhibitory activity of LSE and DIA-2 against α -glucosidase was higher as its IC_{50} was 0.3 $\mu\text{g/ml}$ and 0.7 $\mu\text{g/ml}$ respectively. ASE under the same assay conditions exhibited an IC_{50} value of 136.3 $\mu\text{g/ml}$. The IC_{50} values of all compounds were lower than that of miglitol (651.8 $\mu\text{g/ml}$).

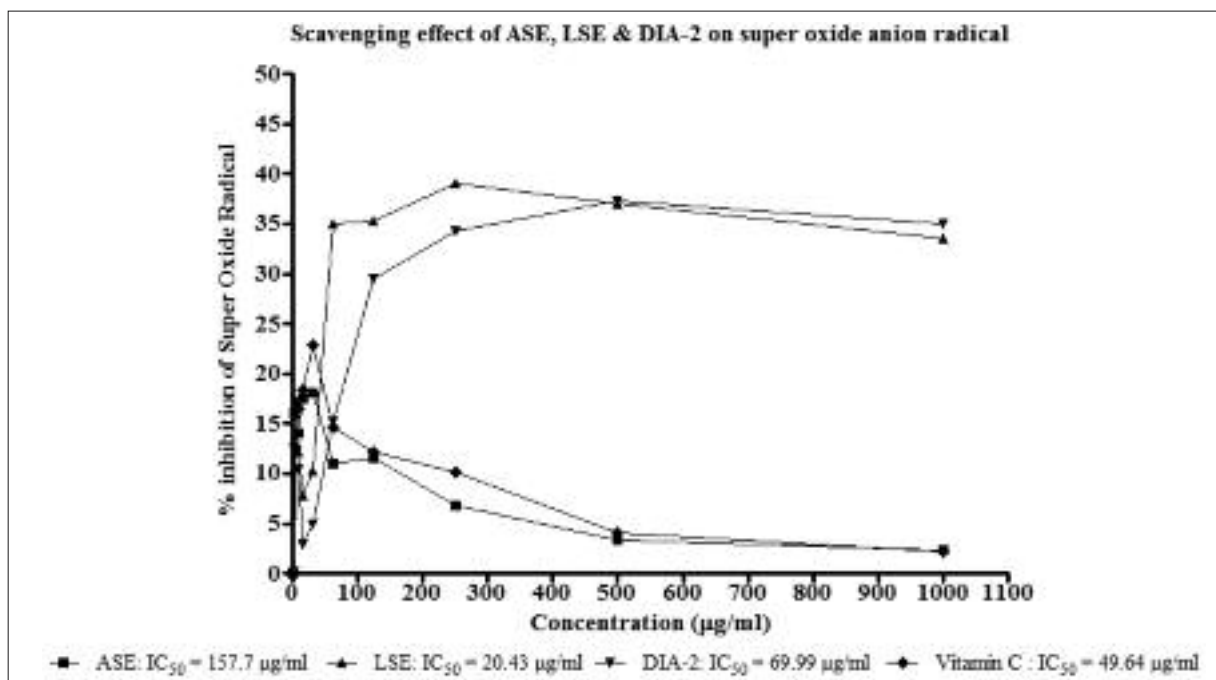


Figure 4. Super oxide radical scavenging effect of ASE, LSE, DIA-2 and AA in an in vitro non-enzymatic system containing PMS/NADH-NBT. The percentage inhibition values shown here are means \pm SEM from experiments performed in duplicates.

Discussion

Medicinal plants have played a significant role in maintaining human health and improving the quality of human life over the centuries. There is an increas-

ing demand for plant derived principles as they are non-toxic and produce less adverse effect¹⁶. Recently, ASE and LSE and their combinations have been investigated for their antihyperglycemic and antioxidant properties¹⁷. Herbal medicines for diabetes are

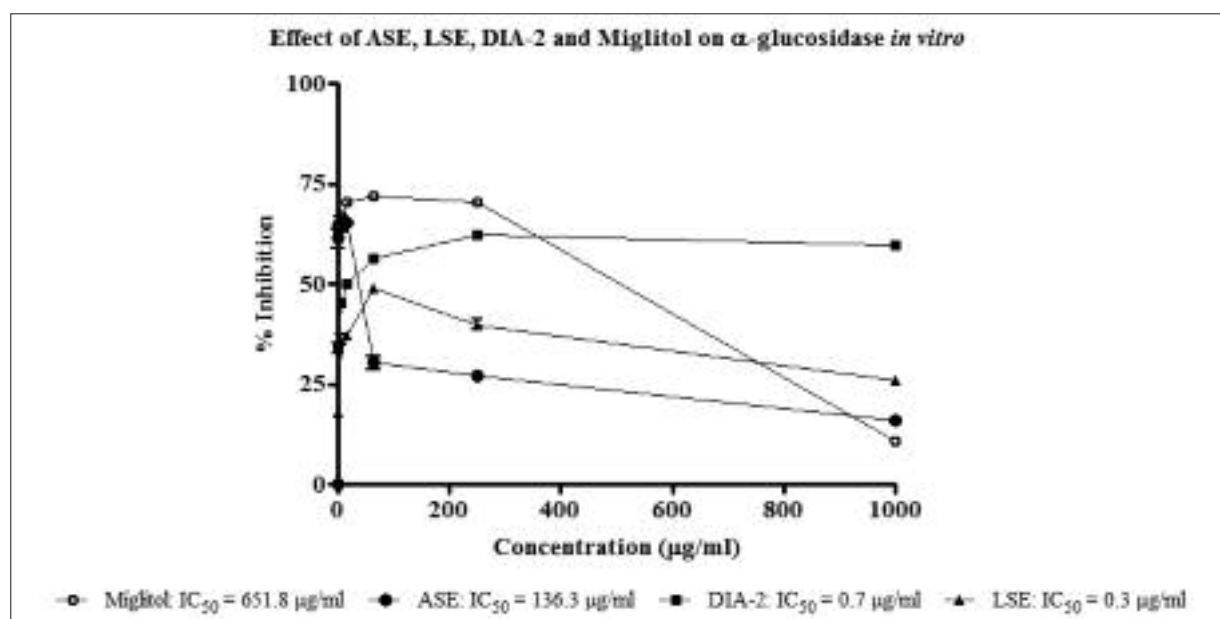


Figure 5. Effect of ASE, LSE and 1:1 combination of ASE and LSE (DIA-2) on α -glucosidase activity *in vitro*. The IC_{50} value (inhibitory concentration) necessary to decrease the absorbance of α -glucosidase by 50% was found to be 0.7 μ g/ml with DIA-2, 651.8 μ g/ml with miglitol 136.3 μ g/ml with ASE and 0.3 μ g/ml with LSE.

by and large mixed formulations containing blood sugar lowering herbs in combination with antioxidants. In this particular study, a combination of *Allium sativum* and *Lagerstroemia speciosa* was developed to combat hyperglycemia and associated free radical damage. Assays like MTT reduction and LDH release have been used to determine cell survival or death¹⁸⁻²¹. Cytotoxicity of ASE and LSE were evaluated primarily to identify their cytotoxic concentrations. Among the extracts, ASE was ~ 6.6 fold cytotoxic than LSE. Based on these results, the herbal mixture (ASE:LSE) was prepared in the ratio of 1:1 and 1:2 and tested for cytotoxicity. Herbal mixture in the ratio 1:2 revealed ~ 4.4 fold cytotoxicity compared to 1:1 herbal mixture. The organosulphur compounds in ASE has been reported to trigger the production of ROS²² and the organosulphur compound, allicin is reported for cytotoxic effect of ASE due to its direct effect on the cell microtubules²³. The organosulphur compounds have also been reported to activate cell apoptosis by virtue of its oxidative inactivation of essential cellular thiol-containing enzymes like glutathione [GSH]²⁴. Sulphydryl reducing reagents (i.e antioxidants) reduces the cytotoxic effects of ASE which have been reported earlier²³. Antioxidant rich polyphenolic compounds (phenols, tannins, flavonoids) present in LSE is comparatively higher than that of ASE and may protect the cells from sulphydryl oxidation²⁵. The cytotoxic effect of 1:1 mixture was found to be less when compared to 1:2 mixture. The high cytotoxic effect of 1:2 mixtures may be possibly due to the higher concentration of ursolic acid, a naturally occurring triterpenoid present in LSE²⁶. Similar findings on cytotoxic effect of *Perilla frutescens* var. *japonica* leaf extract²⁷⁻²⁸ and *Crataegus pinnatifida* var. *psilosa* extract²⁹ was observed due to high concentration of triterpenoids. Based on these findings, 1:1 herbal combination [DIA-2] was considered for further investigations. Cytotoxicity assay also formed the basis to identify the non-cytotoxic dose of DIA-2 and its individual ingredients in the 3T3-L1 preadipocytes and further investigations were made at non-cytotoxic dose range levels.

DIA-2 exhibited synergistic antioxidant and α -glucosidase inhibitory activity compared to its individual component herbs. Hyperglycemia induced metabolic disturbance generates increased production of ROS leading to the development of diabetic complications. In an effort to elucidate the ROS production, super oxide scavenging assay and H₂O₂ induced intracellular ROS production was evaluated. DIA-2 synergistically inhibited super oxide and ROS generation when compared to

the individual extracts. The activity of DIA-2 may be attributed to the individual polyphenolic compounds that are reported to scavenge the free radicals by asset of their antioxidant property³⁰⁻³². The antioxidative³³ potential of ASE differ according to its nature of chemical moieties and standardization process. However, chemical moieties other than organosulphur compounds may contribute to the biological activities³⁴. The inhibitory activity of DIA-2 on H₂O₂ induced ROS production and super oxide radical production may be due to the presence of polyphenols and nonsulfur compounds present in LSE and ASE respectively.

Inhibition of α -glucosidase is one of the therapeutic approaches for delaying carbohydrate digestion, resulting in reduced postprandial glucose. The *in vitro* antihyperglycemic effect of DIA-2 and its individual herbs were assessed by their ability to inhibit carbohydrate hydrolyzing enzyme like, α -glucosidase. Polyphenols have been reported for their α -glucosidase enzyme inhibitory property³⁵. There are some earlier reports to suggest that polyphenolic compounds have both antioxidant and α -glucosidase enzyme inhibitory potential³⁵⁻³⁶. Similarly, a recent study has been reported for antioxidant and inhibitory potential of ASE against α -glucosidase enzyme³⁷. The synergistic effect of DIA-2 to inhibit ROS production and α -glucosidase could be due to ubiquitous polyphenolic compounds and non sulphur compounds.

The study reveals that DIA-2 could be a used as a useful therapeutic candidate in the management of hyperglycemia and hyperglycemia induced oxidative stress. Further *in vivo* studies provide valuable insight to elucidate the mechanism of action and to unravel its role on hyperglycemia and oxidative stress.

Conclusions

In conclusion, combination of *Allium sativum* and *Lagerstroemia speciosa* could be useful therapeutically to manage hyperglycemia and hyperglycemia induced oxidative stress by virtue of their multiple therapeutic activities.

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