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Cardiovascular Toxicity of Oral Antidiabetic Drugs and the Efficacy of Natural Organosulfure Compounds from Aged Garlic Extract (AGE)

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Abstract

The cardiovascular toxicity of oral antidiabetic drugs has become a major concern for patient with type-II diabetes, which demands investigation of the potential natural products to reduce the toxicity of these drugs and improve their efficacy. In the present study, we investigated the metabolic and oxidative stress of oral antidiabetic drugs on cardiovascular system; we evaluated the antioxidative and cardioprotective efficacy of aged garlic (AGE) against drug-induced cardiotoxicity. Metabolic stress of selected classes of antidiabetic drug classes including; biguanides (BG), sulfonylureas (SU), glucagon-like peptide-1 (GLP-1) agonists, dipeptidyl peptidase-4 inhibitors (DPP4-I), thiazolidinediones and sodium/glucose cotransporter-2 inhibitors (SGLT-2) was evaluated by MTT cell viability assay on the H9c2 cardiac cell line. Pre-cytotoxic dose, transition dose and cytotoxic dose of each drug were optimized by MTT assay. The results were further validated by morphological analysis at cardiotoxic doses by giemsa staining and trypan blue dye exclusion assay in a six-well plate experiment. Nuclear alterations and oxidative stress excreted by each drug was evaluated by DAPI and DCFH-DA assay respectively. To evaluate the cardioprotective efficacy of AGE, cell viability assay was performed for each drug co-incubated with 30μM AGE for 48h in MTT plates. The study confirms that SU, DPP-IV and TZD exert metabolic toxicity on cardiac cells by elevating the oxidative stress, morphological, nuclear damage. SGL-2 and GLP-1 were found to be moderately cardiotoxic, while biguanide was found to be cardioprotective. Aged garlic, an antioxidative molecule with stable sulfur compounds was found to exert cardioprotective effect for SU and TZD, it was found to suppress cell death and increase the cell viability by significantly reducing the oxidative stress of antidiabetic drugs. We concluded that aged garlic can be supplemented with anti-diabetic drugs to suppress the cardiotoxic effect of antidiabetic drugs in future drug therapeutics.

Keyword: Diabetes, Antidiabetic drugs, Toxicity, Diabetic cardiomyopathy, Phototherapeutics, Aged garlic, Organosulfur compounds.

Introduction

Diabetes is a chronic metabolic disorder characterized by unregulated blood glucose, which over time promotes cardiac hypertrophy, a condition known as diabetic cardiomyopathy (1). The global diabetic prevalence in 2020 is estimated to be 9.3%, rising to 10.2% by 2030 and 10.9% by 2045 (2). The impact of diabetes mellitus on cardiac function is chronic and silent with prevalence rate 19% to 26% (3-6). With diabetes, Antidiabetic drug-induced cardiotoxicity have found to

induce asymptomatic cardiac complications and worsened the clinical outcome allied with heart failure for patients with diabetes than for those without diabetes. Public health guide to manage hyperglycemia uses first line, second line and third line medication to manage glucose concentration; however no permanent cure is available till date (7). Several clinically approved oral antidiabetic drugs of different classes are only available treatment to regulate the glucose level and prescribed directly after the diagnosis. These drugs are consumed for years to prevent the severity of disease but their chronic accumulation and toxicity to heart tissues is also important to be investigated (8). However, limited information is available for the effect of these drugs on cardiovascular system including cardiac metabolic activity, oxidative stress, hypertrophy, ischemic heart disease, and diabetic cardiomyopathy (9-10). With cardiomyopathy, antidiabetic drug inducing cardiotoxicity has become a major problem in patients suffering with diabetes and eventually increases the risk of cardiac complications and becomes an additional threat of heart attack for diabetic patient (11).

The antidiabetic medications prescribed to the patients with type II diabetes mellitus (TIIDM) has revolutionized in recent years. Metformin is considered to be the first-line therapy if dietary and lifestyle modifications are controlled. Other antidiabetic drugs include sulfonylureas (SUs), thiazolidinedione's (TZDs), dipeptidyl peptidase-4 (DPP-4) inhibitors, sodium–glucose cotransporter 2 (SGLT-2) inhibitors, glucagon-like peptide 1 receptor agonists (GLP-1 RAs), and insulin are the second and third line of medication (11). These six classes of antidiabetic drugs are currently approved for the treatment of diabetes and widely prescribed (12). All of these drugs exerts different molecular mechanism to regulate the blood glucose of diabetic patient. Such as; Metformin works by reduction of gluconeogenesis in the liver and increases the insulin sensitivity **(**13). Sulfonylureas are the second class of drugs commonly prescribed with metformin and work by blocking potassium ion channels of beta cells resulting in depolarization and opining of calcium ion channels. The concentration of calcium ions increases intracellularly causing the release of insulin granules outside the cell (14). Thiazolidinediones exert their antidiabetic effect through a mechanism involved in activation of peroxisome proliferator-activated receptor (PPAR-γ), nuclear receptor that reduce insulin resistance in adipose tissue, muscle and liver (15). Dipeptidyl Peptidase-4 (Dpp-4) Inhibitors increase incretin levels, which inhibits glucagon release and increase insulin secretion. **α**-glucosidase inhibitors inhibit **α**-glucosidase and reduce carbohydrate digestion in intestine (16). Additional to these drugs, anticonvulsant drugs are also prescribed to the patient with diabetic nephropathy (17-18). All of these drugs have different mechanism to keep blood glucose level in moderation but their accumulative effect on the cardiovascular system is still not evaluated mechanistically. The toxicity of these drugs can be hidden when the safety testing is only done on healthy hearts but becomes toxic when in a diseased state. These drugs need to be clinically explored for their toxicity on cardiovascular system to prevent other drug induced severity.

Although the cardioprotective efficacy of medicinal plants has been raised over the years. The possible herb-drug synergistic interaction can proved a potential therapeutic effect with lower toxicity to other organs. Garlic (Allium sativum L.) has been consumed in india from ancient time as a medicinal food for preventing disease and promoting heath (19). Though consuming garlic to attain its therapeutic efficacy also has limitation because of the pungent smell and indigestion. Aged Garlic Extract (AGE) is a derivative of garlic prepared by aging the garlic cloves for twenty months (20-21). Aging of garlic converts reactive organosulfur compounds into stable compounds such as S-allyl cysteine (SAC), Diallyl disulfide (DADS), S-allyl mercapto cysteine (SAMC) etc. These newly formed

allyl compound believe to have exceptional therapeutic benefit as of the raw garlic. Our previous studies suggest the antioxidative and cardioprotective efficacy of aged garlic extract (AGE) over raw garlic. The stable organosulfur compounds present in aged garlic were found to have exceptional therapeutic benefits in our previous studies (21). The present study was designed to evaluate the metabolic toxicity of antidiabetic drugs on cardiovascular system and to investigate the protective herb-drug synergy of AGE with antidiabetic drugs to reduce the cardiovascular toxicity and improve antidiabetic therapeutics for future treatment of diabetes.

Materials and Methods

All chemicals used in the present study were obtained from Sigma Aldrich unless or otherwise stated specifically.

H9c2 cell line maintenance

H9c2 cardiomyocytes derived from rat heart were procured from the National Centre for Cell Science (NCCS), Pune. The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with antibiotics and 10% Fetal Bovine Serum (FBS). The cells were kept in a CO2 humidified incubator (New Brunswick Scientific, USA) containing 5% CO2 and maintained at 37 °C. Cells were subcultured routinely and seeded for experiments or maintained as a running culture with a split ratio of 1:3, up to 10 passages per revived stock.

Sample preparation

Stock and working samples of selected antidiabetic drugs were prepared by dissolving in their respective solvent. Biguanides (50mg/ml), sodium/glucose cotransporter-2 inhibitors (50mg/ml) and dipeptidyl peptidase-4 inhibitors (DPP4-I) (50mg/ml) were solubilized in water. Sulfonylureas (50mg/ml) and glucagon-like peptide-1 (GLP-1) agonists (50mg/ml) were solubilized in ethanol. Thiazolidinediones (50mg/ml) was solubilized in acetone. All the drug samples were sterilized and stored at -2^{0}C before treatment.

Cell cytotoxicity assay

It is a colorimetric assay that quantitate the capacity of reducing 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) dye by living cell mitochondrial reductase enzymes to purple formazan crystals within the cells (23). DMSO is added to solubilize the insoluble formazan crystals into the media and the cell viability is calculated based on the color formation. To measure the cell viability and optimizing doses of the selected antidiabetic drugs along with AGE, cardiomyoblasts were seeded in a 96-welled culture plates at a density of ~ 8000 cells/well. Treatments with increasing concentrations of drugs with and without AGE were given and incubated for 48 h. The induced cells were then stained with 5 mg/ml MTT dye for 3 h after the respective treatment period followed by adding DMSO to solubilize the formazan crystals produced in the cells in the suspension of the culture plate wells. Absorbance of the crystals formed was measured at 570 nm using a microplate reader (BioRad Laboratories, USA). Untreated, control and Treated cell viability was calculated at each dose as (absorbance of treated sample) / (absorbance of control). The results were graphically represented as dose versus percent cellular viability.

Cell viability = Absorbance of sample - Blank / Absorbance of control – Blank

Morphological analysis

H9c2 cardiomyocytes were cultured in a six-well plate for morphological, nuclear, and oxidative stress analysis. ~70% confluent cells was trypsinised and centrifuged at 1500 RPM for 10 minutes. Cell pallet was resuspended uniformly and added in each of the six well and the cells were allowed to adhere for 24 hr. After 24 hr, cells were incubated with different concentrations of aged garlic methanolic extract for 48 hr.

A giemsa staining

Giemsa is a polychromatic stain which stains the nucleus pinkish in colour and the cytoplasm in greyish blue, and used to study the morphology and nuclear integrity of cells. Giemsa has high affinity for the phosphate groups present in DNA and gets attached to the regions of DNA with high adenine–thymine bonding. Cells in different experimental sets were treated followed by rinsing the cells with 1× phosphate buffer saline (PBS) and fixing the cells with 100% cold methanol at - 20°C. 5% giemsa stain (prepared in 1% acetic acid) was then added to each experimental set and kept with gentle shaking 25°C for 15 min. Cells were then observed for morphological alterations at the cellular, cytoplasmic and nuclear level. Images were captured using an inverted microscope at 40× magnification.

B trypan blue dye exclusion assay

Trypan blue stain a diazo dye that specifically stains dead cells blue by binding to the negative charge present on the altered membrane. The stain cannot penetrate the membrane of live cells and hence this technique is named as dye exclusion assay. It is a kind of cell viability assay and in the present study, experimental doses derived from MTT assay were validated using trypan blue dye. Treated cells were trypsinized and resuspended in 1X PBS. 0.4% Trypan blue stain was added in 1:1 ratio and allowed to stand at room temperature for 5 minutes. Total numbers of viable cells were counted and calculated using the formulas mentioned below. The stain was also eluted from the cells using NaOH and quantitated at 595 nm using enzyme-linked immunoassay (ELISA) microplate reader.

Cell viability = Absorbance of sample - Blank / Absorbance of control – Blank

C 4',6-diamidino-2-phenylindoleand or DAPI staining

DAPI is 4',6-diamidino-2-phenylindoleand is a freely permeable blue fluorescent dye. It predominantly stains dsDNA by binding to the A-T rich regions of DNA. This DNA binding results in enhanced florescence in the DAPI filter. Cells grown on coverslips were fixed by methanol fixation and 50 ng/ml DAPI dye was added to the experimental sets. Cells were incubated for 15 minutes at 25°C with gentle shaking in dark. Coverslips were mounted on glass slides and observed under a fluorescent microscope at 100X magnification. The fluorescence intensity was also recorded by spectrofluorometer for the eluted stain from the cells.

Dichloro-dihydro-fluorescein diacetate assay

DCFH stain used to identify and quantify the intracellular H_2O_2 generated upon any stress or oxidative imbalance. This assay is based on the conversion of the nonfluorescent DCFH-DA to 2', 7'- dichlorfluorescein (DCF), a fluorescent compound in presence of excessive peroxides and its increased concentration represents the presence of excessive ROS inside the cells. Cells were fixed with methanol and incubated with 3 µg/ml of DCFH-DA stained in the dark with gentle rocking at room temperature. The stain was eluted and net fluorescence from both detached and adherent cells was measured using a spectrofluorometer at the excitation at 480 nm and an emission wavelength of 530 nm.

Statistical analysis

All experiment were conducted in triplicate and three independent experiments were performed for the quantitative analysis of the data obtained and expressed as mean ± SD. Two-way ANOVA and Student's t-test were done to evaluate the significance of differences in the data obtained. P value less than 0.05 ($p < 0.05$) was considered as statistically significant.

Results and Discussion

In the present study we selected twelve

different drug compounds of six antidiabetic drug classes (Table 1). Cardiotoxic index of these drugs were optimized by incubating the drugs with H9c2 cardiac cell line for 48h in MTT cell viability assay. Morphological and nuclear modulation at optimized doses was then observed. The cell viability and oxidative stress analysis at each doses were observed by DCFH-DA assay.

Table 1. Selected Anti-diabetic Drugs

Class of antidiabetic Drugs	Mechanism of action	Selected Drugs
Biguanide	Inhibition of hepatic glucose production and promotion of skeletal muscle glucose uptake.	Metformin Glucophage
Sulfonylureas (SU)	Depolarization of the beta cell membrane to increase insulin secretion.	Glipizide Glimepiride
Glucagon-like peptide (GLP-1) agonist	Activation of the GLP-1 receptor to augment insulin secretion and inhibit glucagon secretion.	Exenatide Liraglutide
Dipeptidyl peptidase 4 (DPP-IV) inhibitor	Inhibition of endogenous GLP-1 inactivation to augment insulin secretion and inhibit glucagon secretion.	Sitagliptin Vidagliptin
Thiazolidinedione (TZD)	Activation of nuclear receptor peroxisome proliferator activated receptor gamma (PPAR) to increase adiponectin and improve insulin resistance.	Rosiglitazone Pioglitazone
Sodium-glucose-co-transporter inhibitors (SGLT-2)	Inhibition of renal glucose reabsorption.	Canagliflozin Dapagliflozin

a. Biguanides were found to be the least cardiotoxic anti-diabetic drugs.

Biguanide is recommended as the first-line drug for type 2 diabetes mellitus (T2DM) by most international guidelines. The preference for biguanide over other available drugs is based on its efficacy on blood glucose control, tolerability, and safety. Moreover, being derived from the plant *Galega officinalis*, biguanides has a favorable effect on several risk factors on cardiovascular system. In this study, it was observed that biguanides or metformin exerts cardiotoxicity as compared to other classes (24). The cardiovascular outcome of biguanides was found to be safe and did not cause toxic effects on cardiovascular system even at high concentration. Two biguanide compositions namely; Metformin (Met) and Glucophage (Glu) were selected for MTT cell viability assay with concentrations ranging from 5 to 150 µM. The cell viability of cardiomyoblasts treated with increasing concentrations of Met and Glu was derived in comparison to control nontreated cells with 100% cell viability. The IC-50 doses, where cell viability was reduced up to ~50%, were derived for Met and Glu as 100 µM (Fig. 1). No

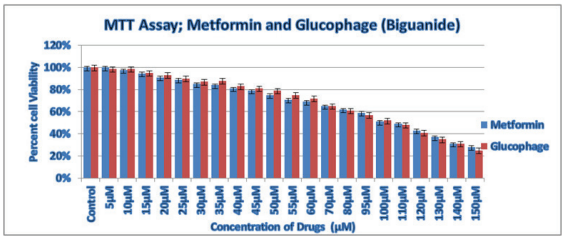

Figure 1. Dose dependent MTT cell viability assay to derive the cardiotoxic doses of Biguanide: a concentrations ranging from 5 µM to 150 µM of metformin and were used and 80 µM was selected as an IC-50 dose. Percent cell viability was compared with respect to Water as a control.

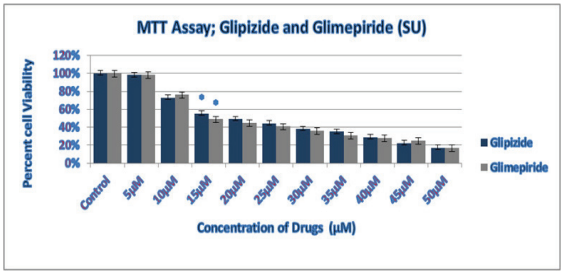

Figure 2. Dose dependent MTT cell viability assay to derive the cardiotoxic doses of Sulfonylurea: a concentrations ranging from 5 μM to 50 μM of glipizide and glimepiride were used and 15 μM was selected as an IC50 dose. Percent cell viability was compared with respect to ethanol as a control.

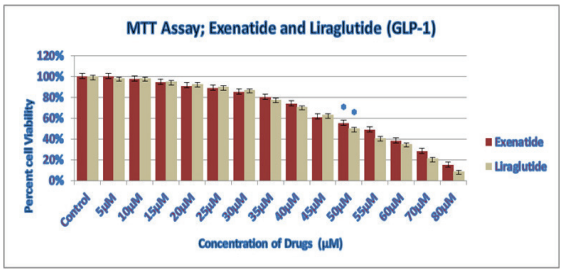

Figure 3. Dose dependent cell viability assay to derive the cardiotoxic doses of GLP-1: concentrations ranging from 5 μM to 80 μM of exenatide and liraglutide were used and 50 μM was selected as an IC50 dose. Percent cell viability was compared with respect to acetone as a control.

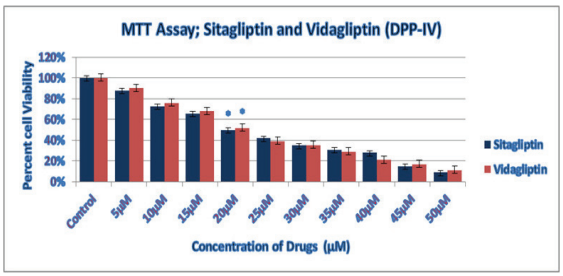

Figure 4. MTT cell viability assay to derive the cardiotoxic doses of DPP-IV: a concentrations ranging from 5 μM to 50 μM of sitagliptin and vidagliptin were used and 20 μM was selected as an IC50 dose. Percent cell viability was compared with respect to water as a control.

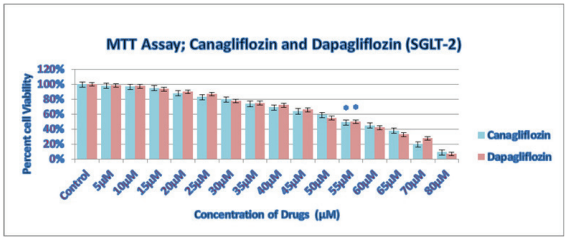

Figure 5. MTT cell viability assay to derive the cardiotoxic doses of SGLT-2: a concentrations ranging from 5 μM to 80 μM of canagliflozin and dapagliflozin were used and 55 μM was selected as an IC50 dose. Percent cell viability was compared with respect to acetone as a control.

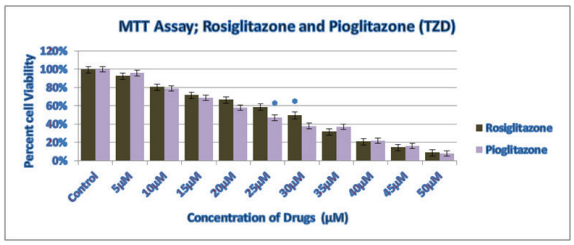

Figure 6. MTT cell viability assay to derive the cardiotoxic doses of TZD: a concentrations ranging from 5 μM to 50 μM of rosiglitazone and pioglitazone were used, 25 μM and 30 μM were selected as IC50 dose respectively. Percent cell viability was compared with respect to acetone as a control.

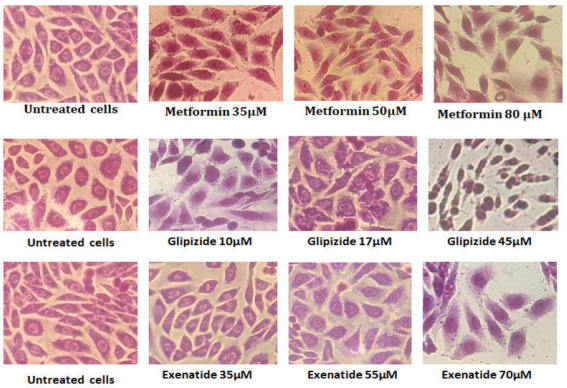

Figure 7. Dose-dependent morphological alteration: Giemsa staining for the cellular integrity of the cells treated with pretoxic, transition and post toxic cardiotoxic doses evaluated by MTT assay.

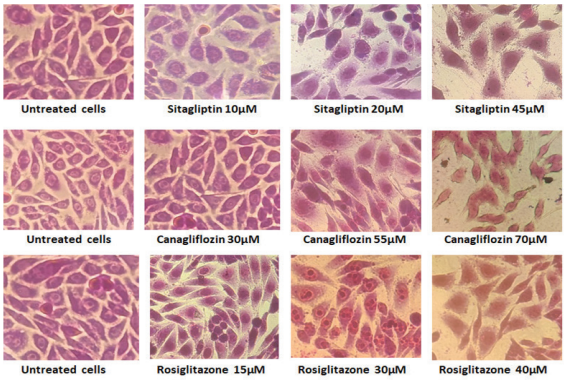

Figure 8. Dose-dependent morphological alteration: Giemsa staining for the cellular integrity of the cells treated with pretoxic, transition and post toxic cardiotoxic doses evaluated by MTT assay.

characteristic stress response was witnessed at pre-cardiotoxic dose (40μM) and transition dose (100μM). A significantly higher number of cell deaths was observed at apoptotic dose (150 μM) (Fig. 12). Approximately ~ 80% cell viability was observed at pre-cardiotoxic dose (40μM), 50% cellular death was observed at transition dose (100μM), and 20% cell viability was found on apoptotic dose (140 μM). These MTT results were further verified by morphological analysis and cell death was observed by trypan blue at these three doses with Giemsa stain. Cellular and nuclear morphology was found to be intact even at the transition dose when compared to control cells (Fig. 7). These evidences suggested that biguanide exerts cardioprotective effect and may improve the cardiovascular safety when supplemented with aged garlic as the viability was improved when MTT assay was conducted with aged garlic at these three doses (Fig.12). The oxidative stress analysis after incubation with antidiabetic drug for 48h was observed by DCFH-DA assay found to be moderately toxic at high concentration (Fig. 10). The synergistic cardioprotective effect of AGE and Met was found to be protective as the cellular viability improved in MTT and trypan blue cell cytotoxicity assays. With these results, it can be concluded that biguanide and aged garlic exert synergistic antidiabetic as well as cardioprotective efficacy (Fig. 9).

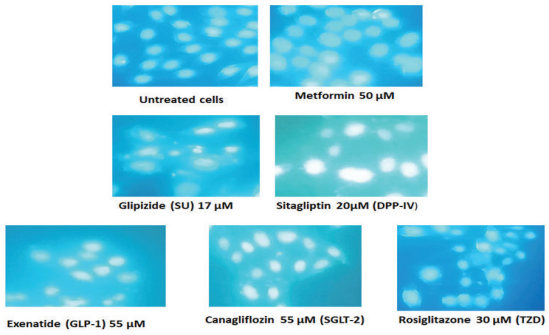

Figure 9. Nuclear alteration: DAPI staining for nuclear integrity of the cells visualized in six well plate treated with cardiotoxic doses of selected anti-diabetic drugs of each classes.

Table 2. Dose Optimization by MTT Assay

Antidiabetic Drugs	Drug Class	IC-50	Pre-cardio-toxic Dose (≤ 80% Viability)	Transition Dose (~ 50% cell viability)	Apoptotic Dose (≥ 80% Viability)
Metformin Melmet	Biguanide	50μM	35μM	50μM	80 μM
Glipizide Glimepiride	SU	17μM	10 μM	17μM	45μM
Exenatide Liraglutide	GLP-1	53μM	35μM	55μM	70μM
Sitagliptin Vidagliptin	DPP-IV	20μM	10μM	20μM	45μM
Canagliflozin Dapagliflozin	SGL-2	55 μM	30 μM	55 μM	70μM
Rosiglitazone Pioglitazone	TZD	27 μM	15μM	30μM	40μM

Table 3. Cardiotoxicity of Anti-diabetic drugs.

Antidiabetic Drugs	Drug Class	Toxicity on Cardiac cell
Metformin Melmet	Biguanide	Least Toxic
Glipizide Glimepiride	SU	Highly Toxic
Exenatide Liraglutide	GLP-1	Moderately Toxic
Sitagliptin Vidagliptin	DPP-IV	Highly Toxic
Canagliflozin Dapagliflozin	SGL-2	Moderately Toxic
Rosiglitazone Pioglitazone	TZD	Highly Toxic

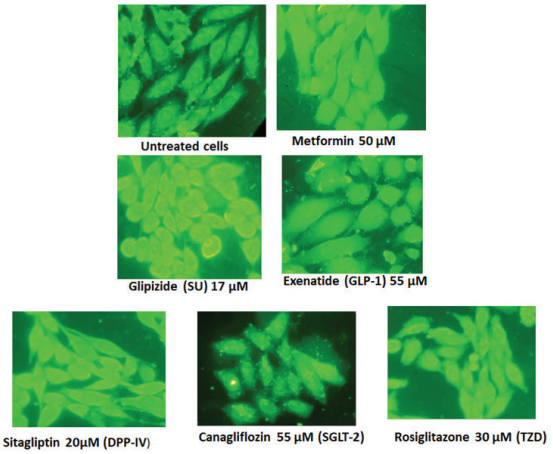

Figure 10. Oxidative stress analysis: DCFH-DA assay for oxidative stress of the cells visualized in six well plate treated with cardiotoxic doses of selected anti-diabetic drugs of each classes.

B. Su, dpp-iv and tzd were found to exert high cardiotoxicity while aged garlic found to have therapeutic potential against su and dpp-iv induced toxicity.

Sulphonylurease (SU) is the second line antidiabetic drugs and prescribed mostly at pre-diabetic and diabetic stage of the patient and therefore its cardiotoxic outcome is essential to evaluate. Glipizide and Glimepiride of SU composition were selected for their cellular cytotoxicity by MTT cell viability assay. The IC-50 doses for both drugs were found to be approximately at ~15 µM concentration (Fig.2). Although a significant cell death was observed even at 10µM concentration. The MTT observations indicated that SU exerted a cardiotoxic effects on cell metabolic activity and viability. These observations were further validated by nuclear and morphological stains by six-well plate experiments (Fig.9). Morphologically, cellular detoriation were observed at 17 µM concentrations, the cell wall and morphological integrity of cardiomyocytes was observed to be scattered as compared to control H9c2 cells, and complete apoptosis was observed at 45 µM concentration (Fig.7). These results indicated the cytotoxic effect of SU on the cardiovascular system. The cardiovascular outcome of sulphonylurease was found to be toxic to the cardiac system even at very low drug concentration. This study suggests that SU significantly reduces the cellular metabolic activity, but the exact mechanism underlying the sulphonylurease toxicity might still not be clear and need to be evaluated on molecular level. However, when the drugs were incubated with aged garlic it significantly improved the cell viability at pre-cardiotoxic transition and apoptotic doses. The cell viability was found to be improved from 70% to 86%, 40% to 72% and 19% to 40% at pre-cardiotoxic, transition and apoptotic dose respectively (Fig.13). Significant improve in cell viability was seen when cells were incubated with aged garlic (30 µM), which provided evidences of the positive effect of stable organosulfure compounds present in AGE to SU induced cardiotoxicity (Fig. 8). In this study we found out the oxidative stress induced by SU drugs supporting the fact that these drugs exert significant cardiotoxicity at low concentration. Supplementing the drug with AGE could improve the cardiovascular safety profile of these drugs. A clear mechanistic understanding of this herb-drug synergy for SU and aged garlic

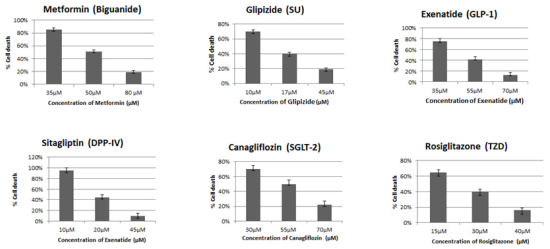

Figure 11. Cell death analysis of selected drug at their precardiotoxic, IC50 and cardiotoxic dose by Trypan blue dye exclusion assay.

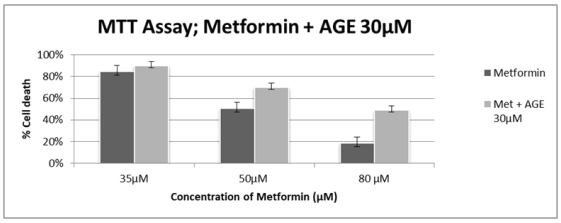

Figure 12. Effect of AGE on percent cell viability in presence of Metformin: MTT assay to investigate the effect of AGE over met induced toxicity was evaluated at different doses.. *p<0.05

may provide the valuable cardiovascular safety outcome in future antidiabetic therapy with antidiabetic and cardioprotective properties.

Dipeptidyl peptidase-4 inhibitors (Sitagliptin and Vidagliptin) medications are either used as single therapy, or in addition to metformin, sulfonylurea, or TZD. These drugs impact postprandial lipid levels. The MTT cell viability IC-50 doses of sitagliptin and vidagliptin were found to be at 20 µM (Fig.4). Although less than 80 % cell death was observed at 10 µM concentration. When cell morphology was observed at Pre cardiotoxic dose (10 µM), transition dose (20 µM) and apoptotic dose (45 µM) (Table 2). Cellular deformities were found to be at transition and cardiotoxic dose. Cellular deformities were observed at 20 µM and 10 µM drug concentration (Fig. 8). Incubation with AGE (30 µM) for 48h was found to improve cellular viability from 95% to 96%, 45% to 63%, and 10% to 35% at pre-cardiotoxic, transition and apoptotic dose respectively (Fig. 15). Aged garlic significantly improved the cell viability at apoptotic dose by approximately three-fold. Synergistic effect of drug with aged garlic extract was found to be protective against cardiovascular drug-induced cardiotoxicity at higher concentration of drug treatment.

Like biguanides, TZDs improve insulin action. Rosiglitazone and pioglitazone were selected for MTT Assay. TZDs are agonists of PPAR and facilitate increased glucose uptake in numerous tissues including adipose, muscle, and liver. Thus, TZDs are not preferred as first-line or even step-up therapy. The IC-50 doses of rosiglitazone and pioglitazone (TZD) were found to be approximately at 27 µM concentration (Fig. 6). Pre-cardiotoxic dose (15 µM), transition dose (30 µM) and apoptotic dose (40 µM) were further selected for the morphological analysis (Fig. 8). Hypertrophy and cell death was observed at 30 µM and 50 µM concentration respectively. Aged garlic incubation was found to improve the cell viability from 65% to 80%, 40% to 75% and 16% to 45% at Pre-cardiotoxic dose (15 µM),

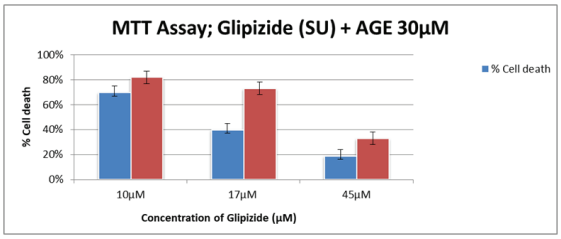

Figure 13. Effect of AGE on percent cell viability in presence of SU: MTT assay to investigate the effect of AGE over SU induced toxicity was evaluated at different doses. *p<0.05

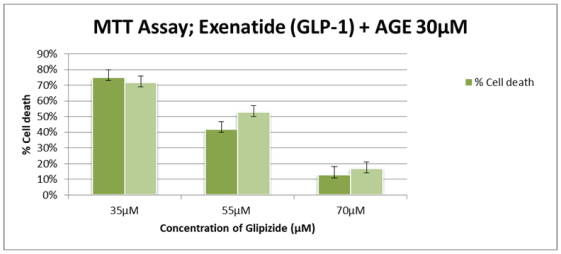

Figure 14. Effect of AGE on percent cell viability in presence of GLP-1: MTT assay to investigate the effect of AGE over GLP-1 induced toxicity was evaluated at different doses.

transition dose (30 µM) and apoptotic dose (40 µM) respectively (14) (Fig. 17). AGE improved the cell viability by approximately two-fold. We concluded that AGE is significantly protective against TZD induced cardiotoxicity.

C. Glp-1 and sgl-2 were found to be moderately cardiotoxic drugs.

Exenatide and Liraglutide were the selected glucagon-like peptide-1 (GLP-1) agonist receptor to investigate the effect on cardiovascular system. These drugs exhibit increased resistance to enzymatic degradation by DPP4. In young patients with recent diagnosis of T2DM, central obesity, and abnormal metabolic profile, treatment with GLP-1 analogs that would have a beneficial effect on weight loss and improve the metabolic dysfunction. The IC-50 doses for both drugs were found to be comparatively greater then SU but not as much as high as metformin. 50 µM doses were found to be the IC-50 dose for both drugs (Fig. 3). Although cell death was increased at higher concentration as only 15 % cell viability was observed at 80 µM concentration. Pre cardiotoxic dose (35 µM), transition dose (55 µM) and apoptotic dose (70 µM) dose of exenatide was selected for morphological analysis in six well plate experiments. No considerable cell death was observed at 35 µM, but significant cell death was observed at 70 µM concentration (Fig. 7). Though GLP-1 agonist was found to be cardiotoxic at high concentration but no significant cell death observed at 50 µM concentration compared to SU. However, when Per cardiotoxic, transition and apoptotic dose of exenatide were incubated with 35 µM AGE for 48h, a slight improvement in cell viability was observed at transition and apoptotic doses but it may not be considered as highly cardioprotective, some of the other studies also indicated the cardiotoxic effect of these drugs on heart (Fig. 11). We concluded that GLP-1 agonist induces moderate cardiotoxicity at low concentration but toxic at high concentration and AGE may not exert cardioprotective effect against GLP-1 induced cardiotoxicity. Further molecular studies are needed to understand the exact mechanism of cardiotoxicity exerted by GLP-1.

Sodium-glucose cotransporter inhibitors are new class of glucosuric agents: canagliflozin, dapagliflozin, were selected to investigate the cellular viability. SGLT-2 inhibitors provide insulin-independent glucose lowering by blocking glucose reabsorption in the proximal renal tubule by inhibiting SGLT2. These drugs provide modest weight loss and blood pressure reduction. The IC-50 doses of

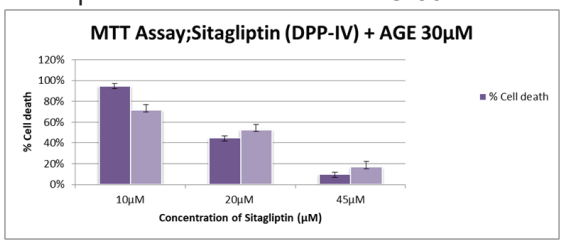

Figure 15. Effect of AGE on percent cell viability in presence of DPP-IV: MTT assay to investigate the effect of AGE over DPP-IV induced toxicity was evaluated at different doses. *p<0.05

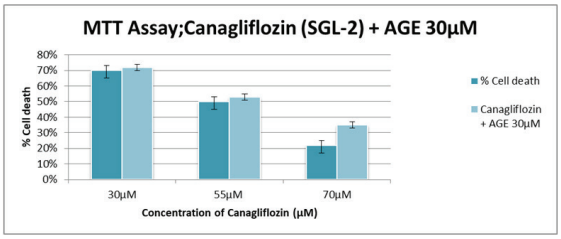

Figure 16. Effect of AGE on percent cell viability in presence of SGL-2: MTT assay to investigate the effect of AGE over SGL-2 induced toxicity was evaluated at different doses. *p<0.05

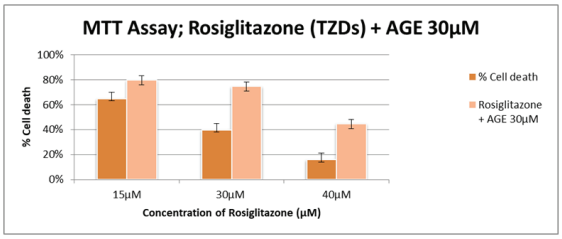

Figure 17. Effect of AGE on percent cell viability in presence of TBA: MTT assay to investigate the effect of AGE over TBA induced toxicity was evaluated at different doses. *p<0.05

canagliflozin and dapagliflozin (SGLT-2) was found to be at 55 µM. Though 80 % of cell death was observed at 30 µM concentration (Fig. 5). Pre-cardiotoxic dose (30 µM), transition dose (55 µM) and apoptotic dose (70 µM) were further selected for morphological analysis. Cellular enlargement or hypertrophy was viewed at transition dose (Fig. 8). Though no significant improvement in cell viability was observed when cells were incubated with aged garlic extract (Fig. 16).

D. Aged garlic extract exert cardioprotective potential through antioxidative activity against drug induced cardiotoxicity.

Aged garlic has been found to have antioxidative and antidiabetic properties. The stable organosulfure compound present in aged garlic has exceptional cardioprotective potential as demonstrated in our previous studies. In this study, we investigated the cardioprotective role of aged garlic against antidiabetic drug-induced cardiotoxicity. In our previous studies, we found that 30 µM is the cardioprotective dose of aged garlic. We incubated the cells for 48h with the pre-cardiotoxic dose, transition dose and apoptotic dose of each drug with 30 µM aged garlic extract. It was found that the cell viability was increased for SU, DPP-IV, and SGT-2 when incubated with aged garlic. Similar results were observed in the morphological and nuclear analysis of H9c2 cell line with six-well plate experiments. Thus, we can conclude that AGE exert a significant cardioprotective effect in diabetes and drug induced cardiotoxicity. Although the exact molecular mechanism is yet to be explored, but stable organosulfure compounds present in AGE may provide dual therapeutic effect on diabetes and cardiovascular system as herb-drug therapy.

Drug-induced toxicity is a major concern for many clinically important drugs. Several antidiabetic compounds were found to cause asymptomatic effects on other organs which led to their post-marketing withdrawal (23). In diabetes, burdening the stress on cardiac tissues (diabetic cardiomyopathy), antidiabetic drugs imposes accumulative stress on the cardiovascular system which ultimately increasing the heart attack ratio among diabetic patients. Currently, assessing cardiovascular safety is a crucial parameter in antidiabetic drug development, and many models have been established to facilitate its prediction to avoid such toxicity. However, cardiotoxicity induced by chronically administered antidiabetic drugs, represents a major problem because cardiovascular stress of these drugs may become evident only after long-term accumulation of the drug or its metabolite (24). Although several natural products have been consumed to reduce hyperglycemia and diabetic complications but limited of those compounds implemented to target specific herb drug therapeutics to improve the bioavailability and efficacy. It has become crucial to investigate the natural products synergistically supplemented with anti-diabetic drugs which do not increase cardiovascular risk but might reduce the risk of chronic heart failure. Therefore, careful selection of herb drug therapy paying particular attention to cardiovascular safety is important in optimizing diabetic therapy. This efficacy and safety of the most commonly prescribed antidiabetic drugs in the context of cardiovascular impacts the future of drug discovery. Our study investigates the cardiotoxicity of conventionally prescribed antidiabetic drugs and the role of aged garlic to reduce cardiotoxicity.

We selected twelve drugs from six antidiabetic drug classes. Biguanide were found to be the least cardiotoxic drug even at high concentration. Being derived from the plant *Galega officinalis*, these drugs exert least cardiac stress. When Biguanide incubated with AGE improved the antidiabetic activity, implying that biguanide has a cardioprotective effect and may improve cardiovascular safety when supplemented with aged garlic (Fig. 12). The oxidative stress analysis after 48 hours of incubation with an anti-diabetic medication was discovered using the DCFH-DA test. In MTT

and trypan blue cell cytotoxicity experiments, the synergistic cardioprotective action of AGE and Met was found to be protective as cellular viability improved with reduced oxidative stress. These observations are also supported by some of the previous data of cardiovascular safety profile for metformin (26). On the other hand SU, DPP-IV and TZD were found exerted a cardiotoxic effects by reducing the cellular metabolic activity. However, when aged garlic were incubated with, SU and TZD significantly improved the cell viability at pre-cardiotoxic transition and apoptotic doses and improved the cellular and nuclear deformities.

SU induced cardiotoxicity was also studied in past research and found to exert left ventricular remodeling (27). AGE improved the cell viability by approximately two-fold. We concluded that AGE is significantly protective against SU and TZD induced Cardiotoxicity (28-29). Synergistic effect of drug with aged garlic extract was found to be protective against cardiovascular drug-induced cardiotoxicity at higher concentration of drug treatment (30). GLP-1 and SGL-2 were found to be cardiotoxic in high concentration (31-33). AGE found to moderately improve the cardiotoxic profile of these drugs.

Natural product have been supplemented with the diet to achieve their therapeutic potential in various disease with less side effects. Though it is also important to keep in mind that diabetes cannot be well-controlled without lifestyle modifications, including proper diet and exercise. Diabetes is a chronic disease that requires a multi-disciplinary approach to optimize blood glucose, blood pressure, and lipid control. Each anti-diabetic drug must be tested for efficacy and therapeutic outcomes before incorporating it into the treatment of diabetic patients. Thus, the toxicity levels of each of the medications were seen and natural plant extracts were used to reduce the cardiotoxic effects. These results suggest that prioritizing drugs on the basis of their cardiovascular protective effects when choosing a treatment for diabetes. Although many antidiabetic drugs have the effect of reducing cardiovascular death and adverse cardiovascular events, it is still unclear which antidiabetic drugs can improve ventricular remodeling and fundamentally delay the process of HF. If drugs can be found to improve ventricular remodeling, it will be of great significance for patients with T2DM with cardiovascular disease (CVD). These results suggest that aged garlic have cardiovascular protective effects should be prioritized when choosing a treatment for diabetes. Herb-drugs synergy can be found to improve cardiac complication, and will be of great significance for patients with T2DM with cardiovascular disease (CVD). The treatment of diabetes mellitus is crucial as the incidence of its occurrence increases. As anti-diabetic medications result in severe cardio toxicity, various remedies need to be formed for better effects. The major anti diabetic drug is considered to be metformin, however combination therapy can also be considered beneficial. Overdoses of these medications may produce major morbidity, with many cases requiring intensive and prolonged medical treatment.

Conclusion

Several antidiabetic drugs have been withdrawn by FDA because of their side effects on other organs, which demand the need of a safer alternative therapeutic. The cardio-protective and antidiabetic role of aged garlic extract has been widely reported, the stable sulfur rich compounds present in AGE were found to be preventive of antidiabetic drug-induced cardiotoxicity. The present study confirms the toxicity of these drugs on cardiomyoblasts. Upon treatment with anti-diabetic drugs, biguanides were found to be safer antidiabetic drugs than sulphonylurease, DPP-IV and TZD. The cardiotoxicity effect was further conformed by altered cellular morphology of H9C2 cardiomyocytes at various concentrations of drugs. SU and DPP-IV were found to be highly toxic drugs among all. The sulfur compounds present in AGE were found

to induce cardio protective potential against antidiabetic drugs and can be supplement with first, second and third line drugs to suppress cardiac stress. The results should be further validated by quantitative assays. These findings suggest the clinical importance of AGE targeting the dual stress responses on the cardiac system of antidiabetic drugs and will not induce any surplus toxicity being a natural and safe compound.

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