7. Discussion

7.1. In vitro evaluation

Inhibition of DPP-4 augments the action of GLP-1 and returns glucose homeostasis toward physiological control levels. Several DPP-4 inhibitors are in development or approved for use in the treatment of T2D. In the course of developing novel DPP-4 inhibitors, the extracts of PM, EJ and GS were tested under identical conditions for their activity against DPP-4. The initial reaction rates of DPP-4 with different concentrations of extracts were analyzed using one-phase exponential decay equation and data indicates that the extracts inhibit DPP-4 in competition to substrate binding sites. Inhibitors that bind tightly to the target are important for the pharmacological activity, as they inhibit enzyme function even if the circulatory free drug is cleared.\textsuperscript{157} The available DPP-4 inhibitors (sitagliptin, vildagliptin and saxagliptin) are potent when compared to our test herbs. Sitagliptin, vildagliptin and saxagliptin have been hailed as a once-daily for the regulation of blood glucose level. However, the extracts used in the present study are available in various marketed formulations and are prescribed twice-daily for diabetes patients. These characteristics may support the potency of extracts in inhibiting DPP-4 when compared with synthetic products.

7.2. Molecular docking

Understanding the complementarity between the drug(s) and the receptor(s) is required to accurately predict the blocking properties of a new compound. Many previous studies have highlighted the importance of DPP-4 inhibition. Many DPP-4 inhibitors are available and many are in development. The aim of the present study is to repurpose the extracts that are well used for the treatment of T2D for AD. To check the validity of our hypothesis, molecular docking was used to investigate the plant constituents on DPP-4. Molecular docking of DPP-4 provides some general principles underlying the affinity
between DPP-4 and the ligands. Constituents whose inhibitory activity is unknown were employed for molecular docking studies in order to find out the molecules that are responsible for biological activity. DPP-4 is a transmembrane glycoprotein known for its cleavage of N-terminal from incretin hormones.\textsuperscript{158} Table 5 and Figures 14 - 16 shows the best six constituents that interacted with DPP-4, and there are also other constituents that bind to DPP-4 with fewer interactions (data not shown). This shows the presence of synergistic effect among the constituents of respective extracts that contribute the anti-diabetic action. All the constituents of GS showed almost same interactions with DPP-4, this might be due to aglycon moiety which forms same interaction in all the constituents. As GS potency was less in \textit{in vitro} evaluation, the other two extracts were chosen for further \textit{in vivo} evaluation.

\textbf{7.3. In vivo evaluation}

AD is an irreversible and devastating neurodegenerative disorder. The major form of AD is sporadic with no known etiology, and accounts 95-97\% of all cases.\textsuperscript{159} Unfortunately, the current treatments for AD only address early symptomatic features of the disease. The five FDA approved drugs are classified into two categories based on their mechanism of actions. Tacrine, donepezil, rivastigmine and galantamine act by augmenting cholinergic neurotransmission by inhibiting acetylcholinesterase,\textsuperscript{160} while memantine, a non-cholinergic drug, acts by antagonizing NMDA receptors.\textsuperscript{161, 162} However, the use of cholinesterase inhibitors is limited due to their adverse peripheral and central cholinergic effects.\textsuperscript{163} Memantine also possess adverse peripheral and central cholinergic effects, but less when compared to cholinergic inhibitors.\textsuperscript{164} Regardless, none of these drugs alter the underlying pathology associated with the disease itself and are mainly palliative and marginally effective. A number of other strategies are currently being investigated. One such strategy is to promote the clearance or prevent accumulation...
of Aβ. This strategy has been found to be effective\textsuperscript{165, 166, 167} and is believed to offer better efficacy than existing drugs.

STZ induced model that recapitulates features of sporadic form of AD has become a widely used rat model for studying the efficacy of pharmacological agents in animals for treating sporadic form of AD. Intracerebral administration of STZ to rats, has been shown to cause Aβ deposition, tau hyperphosphorylation,\textsuperscript{57} and deplete insulin and IGF signaling mechanisms, thereby, induce neurodegeneration and behavioral, cognitive, learning and memory deficits as observed in AD.\textsuperscript{109} Since insulin and insulin signaling mechanisms are important for neuronal survival,\textsuperscript{62} compounds like STZ have been routinely used to induce AD in rats. STZ induced model of AD stands at a special niche among the numerous available plaque or tau pathology models, as this displays a distinct age-dependent cell loss in the brain and related cognitive deficits. Even though several transgenic mice models of AD have been developed, none of these models fully recapitulate the complete spectrum of AD symptoms including plaque and tau pathology, the neuronal loss, and cognitive deficits within a single animal model. For example, many known transgenic AD models display plaque loading at various times of the onset, while others show tau pathology. Likewise, double or triple transgenic mice start to show plaque deposition in the brain at 6 months of age or even at 2 months of age in 5xFAD mice. Therefore, the evaluation of drug candidate for AD has been relied upon plaque models in most cases or tau pathology models in few other cases. Since, STZ induced rat model of AD displays characteristics of neurodegeneration and cognitive deficits, the neuroprotective properties of the extracts of PM and EJ have been tested using this model. Vildagliptin, a DPP-4 inhibitor is a well-established drug for the management of T2D. Previous reports from our lab has shown beneficial effects of vildagliptin in treating dementia of AD.\textsuperscript{45} Therefore vildagliptin has been taken as a standard in this study.
In the present study, STZ has been injected intracerebrally in the rat brain to induce plaque formation. Salkovic-Petrisic and colleagues have reported on the plaque development following three months of STZ injection.\textsuperscript{63} A\textsubscript{\beta}42 is the major species of A\textsubscript{\beta} plaques, which is more prone to aggregate and produce neurotoxicity.\textsuperscript{168, 169} PM and EJ treatment to STZ-treated rats for a period of 30 days resulted in a significant reduction of A\textsubscript{\beta}42 to the level of the sham control group. The findings are consistent with the reports on the DPP-4 inhibitors introduced to the clinic viz., sitagliptin, vildagliptin and saxagliptin, where a significant reduction of plaque load was observed in the brain following treatment.\textsuperscript{43, 44, 45}

DPP-4 inactivates the intestinal hormone GLP-1, an endogenous peptide causing a very short half-life (2 minutes) of the intact hormone.\textsuperscript{170} The concentration of GLP-1 increases following inhibition of DPP-4. In the current study, PM and EJ treatments for a period of 30 days significantly and dose-dependently increased hippocampal GLP-1 levels. An intracerebral injection of STZ did not alter the levels of GLP-1 and is confirmed by the levels of GLP-1 in sham and negative control animals. The increased GLP-1 levels in the treatment groups demonstrate that the reduction of A\textsubscript{\beta} levels might be due to GLP-1 action on its receptors in brain.\textsuperscript{36} Perry and Greig have proposed that GLP-1 binds to APP at its C-terminus, reducing its availability for cleavage, thereby leading to reduction in the formation of A\textsubscript{\beta}.\textsuperscript{171} The suggestion that GLP-1 is involved in learning and memory function\textsuperscript{125} has been confirmed by the behavioral studies. The results are also consistent with the effects of PM and EJ in T2D model, which decelerates the rapid inactivation of GLP-1 through DPP-4 inhibition (data not shown, but published article was attached as Annexure).

Intracerebral injection of STZ leads to hyperphosphorylation of tau protein.\textsuperscript{155} In the present study, PM and EJ treatments not only reduced A\textsubscript{\beta}42 but also reduced total
and p-tau in the STZ-induced AD rats. Tau phosphorylation increases abnormally and accumulates during disease progression in the AD brain.\textsuperscript{172,173} Hyperphosphorylation of tau is cytotoxic and is believed to be a major underlying molecular mechanism for neuronal death in AD.\textsuperscript{174} Present study demonstrate that PM and EJ reduces both total and p-tau levels and reverses the cognitive defects observed in STZ-induced AD rats. In the previous studies, the GLP-1 analogue, (Val8) GLP-1 was shown to mitigate STZ-induced cognitive defects by reducing tau and p-tau\textsuperscript{37}. In the present study, reduction of tau might have also been due to a reduction in A\textbeta levels. This is supported by findings by Oddo et al.\textsuperscript{175} where immunotherapy of A\textbeta reduced tau aggregates. Furthermore, the reduction in the p-tau levels might be due to increased levels of GLP-1 in brain which is consistent with the report of exendin-4, a GLP-1 agonist shown to down-regulate GSK-3\textbeta,\textsuperscript{176} the main kinase that phosphorylates tau.

Intracerebral administration of STZ results in the progressive deterioration of memory and is proposed to be a relevant animal model for SAD.\textsuperscript{152} The observed effect with STZ on learning and memory in animals in this study is also consistent with previous reports.\textsuperscript{177,178} In the present study, RAM and HB tasks were employed for the assessment of learning and memory. An increase in the number of correct choices with decrease in errors in RAM and HB tests with repeated trials demonstrate intact learning and memory function. Following STZ administration, memory deterioration was observed in the non-treated control group of animals. This deficit was reversed, in a dose-dependent manner, following the administration of PM and EJ for 30 days. Additionally, histological examination reveals that this effect may have been due to a reversal of the pathological changes following treatment.

Inflammation is another major hallmark in the AD brain. TNF-\alpha and IL-1\beta, key cytokines produced by activated microglia, play a major role in the pathogenesis of AD.
STZ administration activates microglia in discrete regions of the brain and increases the number of activated astrocytes in the hippocampus. Simultaneously, increased plaque load in the brain also induces microglia activation. Tau phosphorylation and Aβ production is increased by neuro-inflammation. In the present study, elevated levels of TNF-α and IL-1β were efficiently reduced following PM and EJ treatments. This reduction might be due to reduced plaque load following treatment, and is consistent with the previous reports. This reduction in neuro-inflammation might be due to an increase in hippocampal GLP-1 levels, which has been shown with liraglutide, a GLP-1 agonist that reduces neuro-inflammation.