Sains Malaysiana 51(10)(2022): 3383-3399
http://doi.org/10.17576/jsm-2022-5110-22

Antidepressant Potential of Daidzein through Modulation of Endocannabinoid System by Targeting Fatty Acid Amide Hydrolase

(Potensi Anti-kemurungan Daidzein melalui Modulasi Sistem Endokanabinoid dengan Menyasarkan Asid Lemak Amida Hidrolase)

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Received: 17 March 2022/Accepted: 30 May 2022

ABSTRACT

In recent decades, the identification of natural compounds that modulate the endocannabinoid system by fatty acid amide hydrolase (FAAH) inhibition has provided an interesting area of research. Daidzein, which is an isoflavone, has neurobiological activities that are effective against several neurological disorders which include depression. This study aimed to investigate the FAAH inhibitory activity of Daidzein through *in-silico* analysis via Molecular Operating Environment software together with the *in-vitro* FAAH inhibitory assay. Furthermore, the anti-depressive effect of Daidzein (20 mg/kg) was examined via open field test and forced swim test in both male and female mice groups. Finally, the level of depression and stress was measured by the plasma corticosterone level. Molecular docking has shown the probable binding of Daidzein with the FAAH enzyme via its ser-ser-lys catalytic triad. Daidzein binds to the active pocket of FAAH with excellent binding energy of -64.77 Kcal/mol and binding affinity of -11.77 Kcal/mol. The findings reported that Daidzein inhibited the FAAH enzyme with IC_{50} value at 1.3±0.13 µM concentration. The open field test showed that Daidzein had no significant effect on locomotory activity in both male and female groups compared to fluoxetine and Arch-5HT group. Daidzein has significantly decreased the immobility time in forced swim test, which is an indicator of an anti-depressive effect. The corticosterone level that regulates depression was significantly decreased in both male and female Daidzein-treated mice groups. This study highlighted the role of Daidzein as a therapeutic agent for depression via the inhibition of FAAH and modulation of corticosterone levels.

Keywords: Corticosterone; depression; endocannabinoid system; forced swim test; HPA-axis; molecular docking; open field test

ABSTRAK

Dalam beberapa dekad kebelakangan ini, pengenalpastian sebatian semula jadi yang memodulasi sistem endokanabinoid oleh perencatan asid lemak amide hidrolase (FAAH) telah menyediakan bidang penyelidikan yang menarik. Daidzein yang merupakan isoflavon, mempunyai aktiviti neurobiologi yang berkesan terhadap beberapa gangguan neurologi termasuk kemurungan. Penyelidikan ini bertujuan untuk mengkaji aktiviti perencatan FAAH Daidzein melalui analisis *in-silico* melalui perisian Persekitaran Kendalian Molekul bersama-sama dengan ujian perencatan FAAH *in-vitro.* Tambahan pula, kesan anti-kemurungan Daidzein (20 mg/kg) telah diperiksa melalui ujian lapangan terbuka dan ujian berenang paksa pada kumpulan tikus jantan dan betina. Akhirnya, tahap kemurungan dan tekanan diukur dengan tahap kortikosteron plasma. Dok molekul telah menunjukkan kemungkinan pengikatan Daidzein dengan enzim FAAH melalui triad pemangkin *ser-ser-lys*. Daidzein mengikat pada poket aktif FAAH dengan tenaga pengikat yang sangat baik iaitu -64.77 Kcal/mol dan pertalian mengikat -11.77 Kcal/mol. Hasil menunjukkan bahawa Daidzein merencat enzim FAAH dengan nilai IC_{50} pada kepekatan 1.3±0.13 µM. Ujian lapangan terbuka menunjukkan bahawa Daidzein tidak mempunyai kesan yang signifikan terhadap aktiviti lokomotor pada kedua-dua

kumpulan jantan dan betina berbanding kumpulan fluoksetin dan Arch-5HT. Daidzein telah mengurangkan dengan ketara masa pergerakan dalam ujian berenang paksa yang merupakan penunjuk kesan anti-kemurungan. Tahap kortikosteron yang mengawal kemurungan telah menurun dengan ketara pada kumpulan tikus jantan dan betina yang dirawat Daidzein. Kajian ini menekankan peranan Daidzein sebagai agen terapeutik untuk kemurungan melalui perencatan FAAH dan modulasi tahap kortikosteron.

Kata kunci: Dok molekul; kemurungan; kortikosteron; paksi HPA; sistem endokanabinoid; ujian berenang paksa; ujian lapangan terbuka

Introduction

Depression is a multifactorial psychiatric and neurological disease (Beck et al. 2014). It is one of the most widespread mood disorders that is associated with significant disability and impaired quality of life (Lim et al. 2018). The advancement in basic and clinical investigations enables the detailed molecular mechanism of depression (Alexopoulos 2019). The monoamine theory predicted that the underlying mechanism of depression is based on decreased levels of serotonin, norepinephrine and dopamine in central nervous system (Schildkraut 1965). This hypothesis resulted in the development of various anti-depressant drugs such as selective serotonin reuptake inhibitors and serotonin norepinephrine reuptake inhibitors. The existing anti-depressants face challenges such as refractoriness, complete retrieval, reversion, slow onset of action, sexual dysfunction, weight gain as well as cardiovascular and gastrointestinal problems (Khan et al. 2014; Rosenzweig-Lipson et al. 2007). A number of hypotheses have been put forward to explain the pathophysiology of depression; however, the endocannabinoid system dysregulation has recently been reported to have a noticeable role in depression (Yin et al. 2019). Thus, the current situation demands to put much efforts in investigating novel anti-depressants with better efficacy and safety by regulating endocannabinoid system.

The endocannabinoid system is comprised of cannabinoid receptors such as CB1 and CB2 receptors. Their endogenous ligands are anandamide (arachidonoylethanolamide, AEA), 2-arachidonoylglycerol (2-AG) and certain enzymes that are involved in endocannabinoid synthesis and metabolism (Chadwick et al. 2020). CB1 and CB2 are G-protein-coupled receptors. The endocannabinoid system regulates several physiological processes that include cognition, appetite and pain perception (Cristino et al. 2020). The control of emotion and motivation is particularly important for possible involvement of this system in pathology of depression (Zou & Kumar 2018). The endocannabinoid produces its effect by binding to CB1 and CB2 receptors, which is further inactivated by uptake and/or degradation. The endocannabinoid is degraded by two specific enzymes, namely fatty acid amide hydrolase (FAAH) and monoacylglyceride lipase (MAGL) (Toczek & Malinowska 2018). Anandamide is degraded by FAAH into ethanolamine and arachidonic acid, whereas 2-AG is degraded by MAGL into glycerol and arachidonic acid (Bari et al. 2006).

Endocannabinoids work as a retrograde messenger. The endogenous anandamide is released from dendritic region in a non-vesicular calcium-dependent way by acting on CB1 receptors that are located in presynaptic axons in brain regions such as prefrontal cortex, hippocampus, amygdala, nucleus accumbance and striatum (Castillo et al. 2012). The synaptic anandamide binds to CB1 receptor couples via three important pathways: inhibitory effect on Adenylyl Cyclase, K+ and Ca2+ channels, and positive effect on mitogenic activation protein kinase (MAPK). The inhibition of adenylyl cyclase ultimately results in a decrease in cAMP content that reduces enzymes, particularly protein kinases. Protein kinases have an important role in modulating different ion channels and releasing monoamine neurotransmitters (Wilson & Nicoll 2002). Hence, the endocannabinoid system regulates the synaptic transmission of both excitatory and inhibitory pathways by modulating the release of neurotransmitters (Katona & Freund 2012). Conclusively, the effect of anandamide depends upon the localization of cannabinoid receptors within the inhibitory or excitatory neuronal circuits (Wilson & Nicoll 2002). The evidence from several studies in the last two decades has proven that the hypofunction of endocannabinoid system can produce the symptoms of depression. Therefore, this system may present a new therapeutic approach in managing depression (Vinod & Hungund 2006).

In all living organisms, the combination of sex-specific genetics, hormones and epigenetic produces

diverse type of *in-vivo* environment for males and females. Different genders modify the pharmacodynamics and pharmacokinetics responses of drugs. Several clinical and community-based epidemiological studies reported that women are more prone to depression as compared to men (Gaynes et al. 2005; Kessler 2003; Kuehner 2003). Additionally, there is a difference in response of drugs by men and women towards anti-depressant drugs (Kornstein et al. 2000). Therefore, sexual dimorphism must be considered while conducting experiments in order to find the most suitable anti-depressant.

There are several compounds available that interact with different stages of endocannabinoid transmission and degradation, hence making it easy to study the role of endocannabinoid in physiological and pathological conditions (Pflüger-Müller et al. 2020). While investigating the role of compounds for their anti-depressant effects, two classes of compounds which are FAAH inhibitors and CB1 agonist are used. Hence, compounds that specifically modulate the FAAH activity with the least side effects are the best choice to treat depression.

Currently, compounds obtained from natural products are perceived as a safe alternative approach to discover novel multipotent drugs against depression (Uddin & Kabir 2019). Among these compounds, isoflavones are an important class because of various beneficial biological activities (Alshehri et al. 2021). Daidzein, which is an isoflavone, abundantly exist in soybeans and other legumes, and these soy-based food are regularly consumed in Asian countries (Ronis et al. 2020). Daidzein is chemically a 4,7-hydroxy-isoflavone and exhibits a wide range of therapeutic activity against several diseases that include cancer (Guo et al. 2004), diabetes mellitus (Hintz & Ren 2004), heart diseases (Wang et al. 2003), Alzheimer's disease (Wei et al. 2019; Wu et al. 2021) and depression (Gu et al. 2006). Daidzein plays a vital role in neurological disorders via several mechanisms. It affects hippocampal neuron proliferation that is the expression of brain-derived -neurotrophic factor (Pan et al. 2012) and activates cholinergic system to improve memory (Ko et al. 2018a). It involves phospho-cAMP response element binding protein or brain-derived neurotropic factor (p-CREB/BDNF) pathways to improve cognitive functions (Ko et al. 2018b). Daidzein has also shown promising effects against neurodegenerative diseases by decreasing beta-amyloid and beta-amyloid-induced neurotoxicity in neuronal cells (Choi et al. 2013). Due to its phytoestrogenic structure, Daidzein has profound effects on the brain; therefore, the neurobiological effects of Daidzein provide a vast area of research in neurological diseases. Therefore, Daidzein was explored for its endocannabinoid modulating activity specifically for the treatment of FAAH as there were reports of endocannabinoid hypofunction in the pathology of depression (Micale et al. 2015). Hence, it was hypothesized that Daidzein might modulate the endocannabinoid system by interfering with the cellular uptake of anandamide or by inhibiting FAAH. This study not only aimed to explore Daidzein as a selective inhibitor of FAAH through *in-silico* and *in-vitro* assays, but also aimed to further study its anti-depressive effects on behavioural tests and plasma corticosterone levels in both male and female mice models of depression.

Materials and Methods

Chemicals and laboratory animals

Daidzein (D251600-25MG), Fluoxetine hydrochloride (F132-50MG), Arch-5HT (A7357-25MG) were obtained from Sigma-Aldrich USA. Balb/c mice were purchased from National Institute of Health in Islamabad. The average weight of both male and female mice was between 30 and 35 gram. The virgin female mice were used irrespective of their estrous cycle stage. The animals were kept under standard environmental conditions (25 ± 2 °C) in a 12-hour light/dark cycle with unobstructed access to food and water. The mice were kept in plexiglass cages in a group of four. Initially, three different doses of Daidzein (10, 20, and 30 mg/kg) were screened and the most effective dose that was 20 mg/kg was then selected for further study. Fluoxetine (10 mg/kg) was used as a positive control, while Arch-5HT that is a known endogenous FAAH inhibitor was used at a dose of 5 mg/kg as the standard FAAH inhibitor. Daidzein was administered for 14 days intraperitoneally (Śliwa & Macura 2005) to examine the chronic effect of the compound as well as to compare it with the standard anti-depressant drug fluoxetine (Cryan et al. 2005). Its effect on serum corticosterone levels was also observed.

Experimental protocol

in-silico screening of Daidzein was performed to predict the possible binding sites and the extent of inhibiting FAAH enzyme. Daidzein was screened for FAAH inhibitory effect by using enzyme-linked immunosorbent assay (ELISA) kit. For *in-vivo* testing, a total of 64 mice were used, including 32 male mice and 32 female mice. Both male and female mice were divided into four

groups and each group consisted of 8 mice which were categorized into vehicle group, Arch-5HT (Arch-5HT) treated group, Daidzein treated group and Fluoxetine treated group. All the drugs were administered chronically for 14 days through intra-peritoneal (i.p) route (Vázquez-Palacios et al. 2005). The locomotory effect was measured by open field test, whereas the antidepressant effect of Daidzein was examined by forced swim test after 14 days of drug administration. Both behavioural tests were performed on the same animals. Finally, the plasma corticosterone levels of treated mice were measured.

COMPLIANCE WITH ETHICAL STANDARDS

All experiments were performed according to the protocols of Research Ethics Committee (Ethical Approval no. PHM.Eth/29CF-M02-052020) of the COMSATS University, Abbottabad Campus in accordance with the rulings of the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals 2011).

in-silico DOCKING ANALYSIS

Molecular docking was performed to find out the binding and inhibitory activity of Daidzein with FAAH enzyme. Molecular operating environment (MOE) software version 2015.10 from Chemical Computing Group Inc was used for the docking. The crystallographic X-ray structure of FAAH coupled with inhibitor PF-750 (PDB ID: 2VYA) was downloaded (PDB format) from the protein data bank. This crystallographic structure of humanized rat (h/r) FAAH illustrated the inhibitor sensitivity profiles of human FAAH (Mileni et al. 2010). The primary amino acid structure of rat and mouse FAAH are 91% identical to each other, whereas the human FAAH has the structure similarity of 84% with mouse and 82% with rat FAAH (Mileni et al. 2008). The protein was protonated, and the energy was minimized using the forcefield Amber10. The water molecule and unwanted chain were removed, hydrogen bonds were added, and the protein structure was saved for docking (Kumar et al. 2012; Zhang et al. 2017).

The ligands (Daidzein, PF-750 and Arch-5HT) SMILES were copied from PubChem and pasted in the MOE to get 2D structures. Subsequently, they were converted to 3D structures by adding partial charges via MOE and the energy was minimized. The structures were saved in the database for further process (Borkotoky et al. 2016).

Rigid receptor docking (RRD) with the application of Proxy triangle protocols was used for molecular docking that was implemented in MOE. The docking scores were presented in negative energy terms, where lower binding free energy means a high affinity for the binding (Lensink et al. 2007). The parallel re-docking was performed to validate the docking protocol and RMSD value of co-crystalized ligand was calculated.

FAAH INHIBITORY SCREENING ASSAY

FAAH assay was performed by FAAH inhibitor screening assay kit with the catalogue number of 10005196 (Manufacturer Cayman chemicals, USA). All the procedures were performed according to instructions provided by the kit. This assay kit is based on fluorescence method for screening FAAH inhibition (Almukadi et al. 2013; Bruno et al. 2014). The plate was measured at excitation wavelength of 340-360 nm and an emission wavelength of 450-465 nm. The percentage inhibition for each sample was calculated using the formula as follows:

$$\% \text{ inhibition} = [(\text{Initial activity} - \text{Sample})/\text{Initial activity}] \times 100$$

in-vivo Analysis

In-vivo analysis of drugs was carried out initially by behavioural tests. Forced swim test and open field tests were performed to evaluate anti-depressive and anxiolytic activity, respectively. The male and female mice were transferred to behaviour room one hour prior to the test in order to familiarize the rodents to the conditions of testing room.

Open field test

The test was performed to check the locomotion, anxiety, and exploratory behavior. The test procedure was similar to that of Gould et al. (2009) with slight modifications. A mouse was placed into an open field apparatus (an iron alloy box with diameters of 50 cm length × 50 cm width × 38 cm height) for a habituation time of 60 min. Then, the test compounds were injected with i.p and then placed again in the open field apparatus. The locomotion of mouse was recorded for 60 min. After completing the test, the open field apparatus was properly cleaned with 70% ethanol. The video was analyzed for the distance covered as well as time spent in center and periphery of the open field. The effect of all drugs was compared with the vehicle control group.

Forced Swim test (FST)

The FST was performed as mentioned previously by Can et al. (2012). A tank with the height of 30 cm and a diameter of 20 cm was filled to a desired level with water being set at room temperature (23-25 °C). The water level was maintained at 15 cm from the bottom of the tank. The video recording was started before placing the animal into the water tank. The mouse was held from its tail and was then released slowly into the water tank to prevent the head of the mouse from submerging into the water. The mice were left in water tank for 6 min and the recording was stopped. The animals were removed from water tank and dried properly before transferring back to their cage. The time that each mouse spent mobile was measured by blind observer using a stopwatch. The measured time was subtracted from the total test time. This time was considered as immobility time.

CORTICOSTERONE MEASUREMENT

Immediately, after the completion of FST, blood was collected by cardiac puncture injection after the mice were anesthetized with ketamine (Parasuraman et al. 2010). The collected blood was centrifuged at 14000 rpm for 3 min immediately. The separated serum was collected after centrifugating into Eppendorf tube and was stored at -20 degree centigrade till further use.

Corticosterone, which is the major indicator of stress, was measured quantitatively from plasma samples obtained from the treated mice. The assay was performed by DetectX Corticosterone Enzyme immune assay kit with the catalogue number KO14-H5 (Manufacturer ARBOR Assays). All the procedures were performed according to the instructions provided by the kit (Melo de Carvalho 2015).

STATISTICAL ANALYSIS

All the data were expressed as mean ± standard error of mean (SEM) while 'n' is the number of rodents in each group. Statistical analysis of the results was performed by 'Graph Pad Prism 8.0.1' software and the results were considered significant only if the '*p*' value was less than 0.05. One-way ANOVA was applied, followed by Bonferroni post-test.

RESULTS

DAIDZEIN POSSIBLE BINDING AND INHIBITION OF FAAH ENZYME

Daidzein was studied by molecular docking against FAAH in comparison with the standard FAAH inhibitors, namely PF-750 and Arch-5HT (Johnson et al. 2011) to examine the attraction, specific binding mode and presumed interactions with the active site of the enzyme. The binding site of PF-750 with FAAH crystalline structure was parallel with Daidzein as both of them interacted through Ser-241 (as shown in Figure 1) that was responsible for the enzymatic hydrolytic activity of FAAH.

The docking simulation (Figure 1(a)-1(e)) showed that the Daidzein had promising interaction with the enzyme active site Ser-241 as shown in Figure 1(b). Arch-5HT had a high docking score of -9.8445, followed by PF-750 with -8.5750 and Daidzein with -6.2772. The binding position of PF-750 and Arch-5HT are shown in Figure 1(a) and 1(c). Daidzein and Arch-5HT had high binding free energy (-64.77 Kcal/mol and -75.00 Kcal/mol) compared to PF-750 (−54.46 Kcal/mol) which served as a threshold of the standard. Docking simulations showed that Arch-5HT had the highest score (-9.8445).

Daidzein has a docking score of -6.2772, which was a bit lower than PF-750 and Arch- 5HT (docking score of -8.5750 and -9.8445, respectively); however, the binding affinity of daidzein (11.74 Kcal/mol) was more than Arch-5HT (9.97 Kcal/mol) and PF-750 (8.65 Kcal/mol). Daidzein complex formed with the active site (Ser-241) of FAAH enzyme envisaged the capacity of the compound to obstruct FAAH activity. The colored three-dimensional structures of daidzein and PF-750 in the active pocket of FAAH enzyme are shown in Figure 1(d). Moreover, the three-dimensional structure of Daidzein and PF-750 in active domain of FAAH is shown in Figure 1(e). The data are summarized in Table 1.

in-vitro FAAH INHIBITION BY DAIDZEIN

Daidzein inhibited FAAH in a dose dependent manner with the IC_{50} value of 1.3±0.13 μM concentration. The percentage of FAAH inhibition increased with the increasing dose of Daidzein as shown in Figure 2. Moreover, 100 μM concentrations of Daidzein produced an equivalent response to the standard FAAH inhibitor, JZL-195 at a concentration of 10 μM. JZL-195 is a standard FAAH inhibitor which is available with the kit.

EFFECT OF DAIDZEIN ON LOCOMOTORY ACTIVITY

Daidzein was explored for its psychostimulant activity via open field test. Daidzein treatment showed no significant effect on locomotion and exploratory behaviour. The distance travelled by male mice fluoxetine

(23.46±4.0 m) and Arch-5HT treated (22.6±3.8 m) groups were the same as compared to vehicle (19.08±3.15 m) and daidzein (17.57±3.9 m) treated groups, thus showing no significant difference (Figure 3(a)).

Figure 1(a):

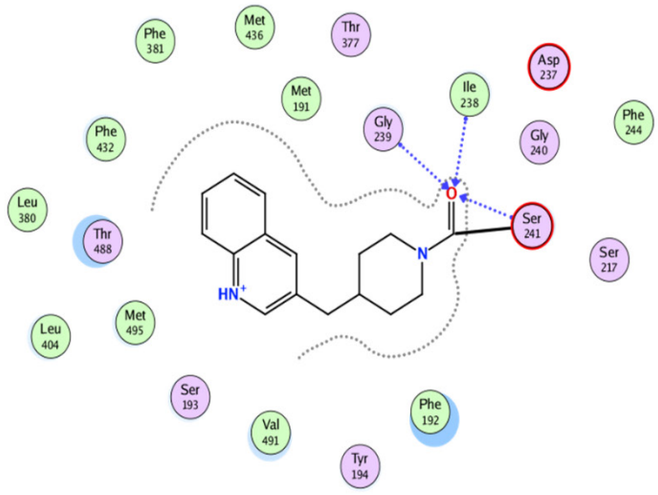

Figure 1(b):

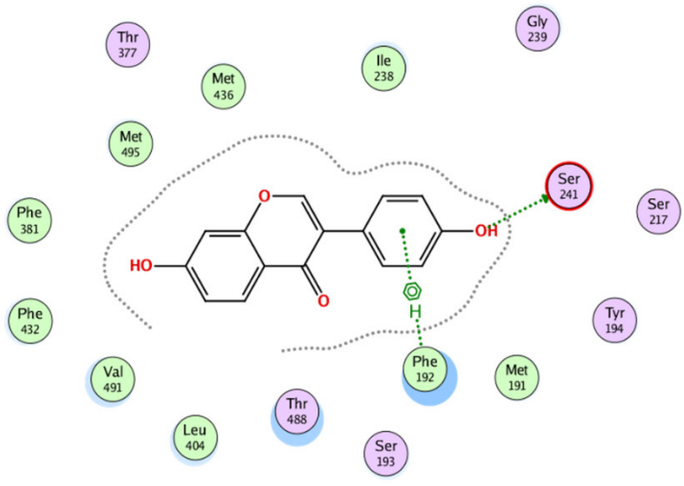

Figure 1(c):

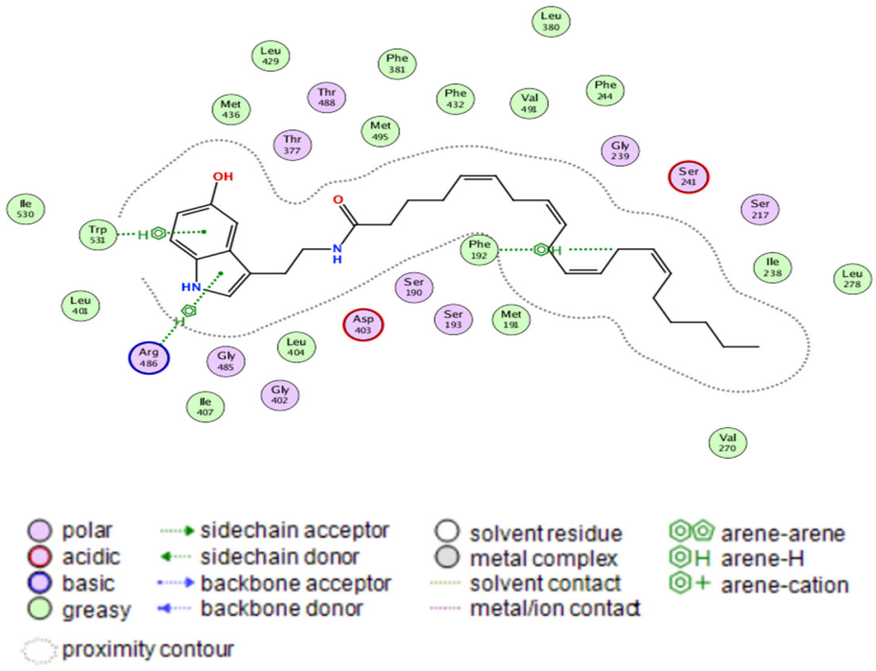

Figure 1(d):

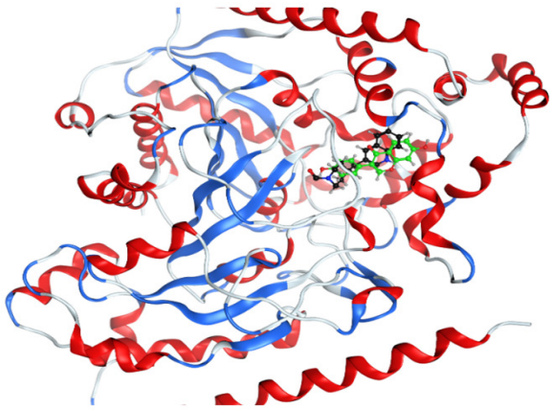

Figure 1(e):

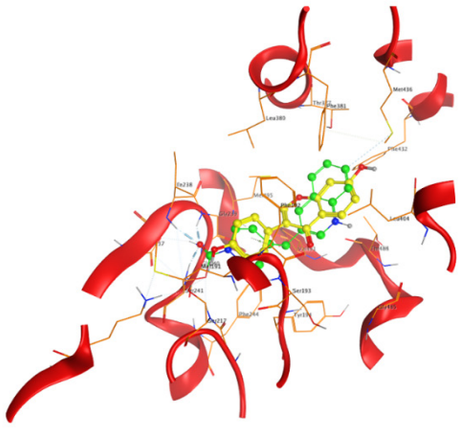

FIGURE 1. *in-silico* docking analysis of Daidzein (a) Interaction of the PF-750 with the residue of FAAH active site (reference) in two-dimensional (2D) plot, (b) 2D structure of Daidzein with the residue FAAH active domain showing the bond with ser 241, (c) 2D structure of Arch-5HT with the residue of FAAH active site, (d) Three dimensional (3D) structure of the Daidzein (green) and PF-750 (black) in the active pocket of the whole FAAH enzyme, and (e) 3D structure of the Daidzein (green) and PF-750 (yellow) in the active domain of the FAAH enzyme

TABLE 1. Docking score and binding free energy (Kcal/ mol) of the Docked compound (Daidzein, Arch-5HT and PF-750)

Compound	Docking score	Binding energy (Kcal/mol)	Binding affinity Kcal/mol
Daidzein	-6.2772	-64.77	-11.7741
Arch-5HT	-9.8445	-75.00	-9.9742
PF-750	-8.7550	-54.46	-8.674

The same behaviour could be seen in female mice group. However, the male mice travelled relatively longer distance compared to female mice. In female mice group, the average distance travelled by Arch-5HT group (17.57±3.9 m) was a bit longer than vehicle (16.95±3.9 m), daidzein (14.86±3.6 m) and fluoxetine treated groups (14.47±3.4 m); however, no significant difference was detected among the groups (Figure 3(b)).

EFFECT OF DAIDZEIN ON STRESS AND DEPRESSION

The role of Daidzein on stress and depression was measured by forced swim test. After14 days of drug treatment, the forced swim test was performed for an hour after the last dose was injected. The Daidzein treatment among male mice (35.5±7.6 s) led to the decreased level of depression with less immobility time as compared to vehicle treated group (91.0±7.2 s; ***p<0.001). The immobility time in forced swim test was also decreased by fluoxetine treatment (47.1±7.7 s; ***p<0.001) and Arch-5HT treatment (51.8±7.6 s; **p<0.01) as compared to vehicle groups (Figure 4(a)).

The female group of Daidzein treatment (34.2±0.5.04 s) showed a decreased level of stress and immobility time as compared to vehicle treated group (89.6±7.04sec; ***p<0.001). The daidzein treatment led to similar levels of decreased immobility for fluoxetine treatment (43.6±6.7 s;***p<0.001) and Arch-5HT treated (50.1±8.1 s;**p<0.01) female mice groups as compared to vehicle group (Figure 4(b)).

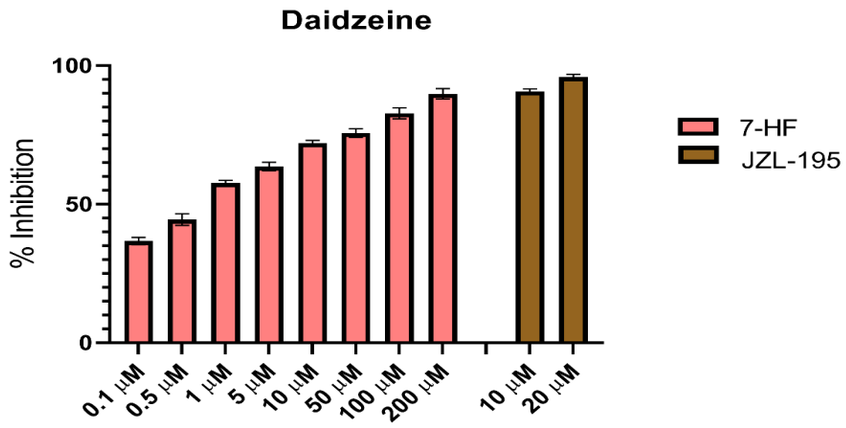

FIGURE 2. Graph showing the inhibition of FAAH enzyme by Daidzein. The figure depicting the dose dependent response of Daidzein from 0.1 µM to 200 µM concentrations. Data are shown as mean ± SEM. JZL195 (10 µM and 20 µM) was used as standard FAAH inhibitor. IC_{50} value is 1.3±0.13 µM

Figure 3(a):

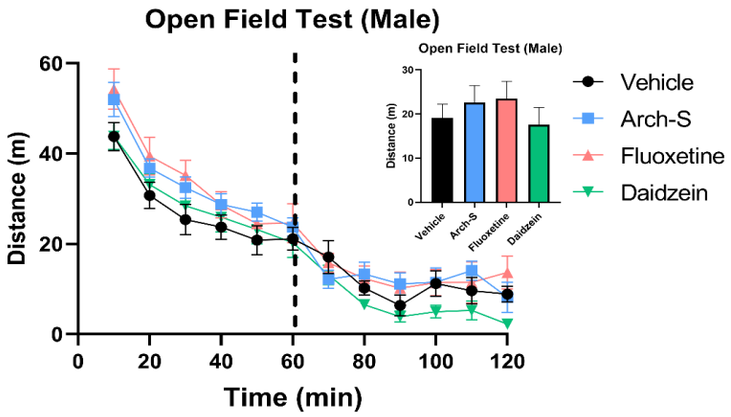

Figure 3(b):

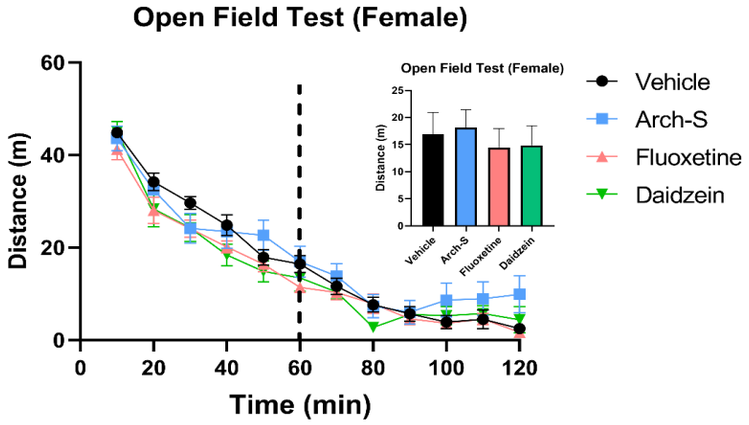

FIGURE 3. Graphs showing the distance covered by mice in open field test of vehicle, daidzein, Arch-5HT and fluoxetine treated groups. The distance in meters was measured for 60 min before the drug administration and 60 min after the drug administration. One way ANOVA was used whereas the number of mice in each group is 8. Data are presented as mean ±SEM (a) The open field test results of male mice group and (b) The open field test of female mice group. There was no significant difference among the treated groups in both male and female mice

EFFECT OF DAIDZEIN ON PLASMA CORTICOSTERONE LEVEL

After conducting forced swim tests, plasma corticosterone levels in male and female mice groups were measured. The male mice which were exposed to forced swim test and injected with vehicle (9.97±1.46) showed significantly elevated level of corticosterone in plasma as compared to Daidzein (5.7±0.4 ng/mL;**p<0.01), fluoxetine (6.5±0.3 ng/mL;*p<0.05) and Arch-5HT treated groups (6.5±0.7 ng/mL;*p<0.05). However, there was no significant difference among Daidzein and Arch-5HT treated groups (Figure 5(a)).

For female mice group, Daidzein treatment (5.4±0.85 ng/mL) significantly reduced the level of plasma corticosterone levels as compared to vehicle treated group (9.97±1.4 ng/mL; ***p<0.001). There was a significant decrease level of corticosterone in Arch-5HT treated group (6.02±0.2 ng/mL; **p<0.01) and fluoxetine treated group 6.47±0.3 ng/mL; *p<0.05) that were relative to vehicle group (Figure 5(b)).

Figure 4(a):

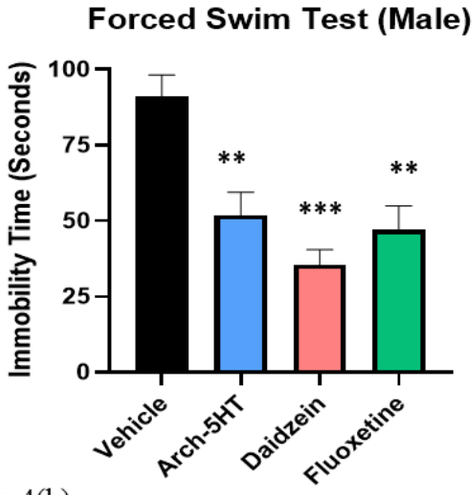

Figure 4(b):

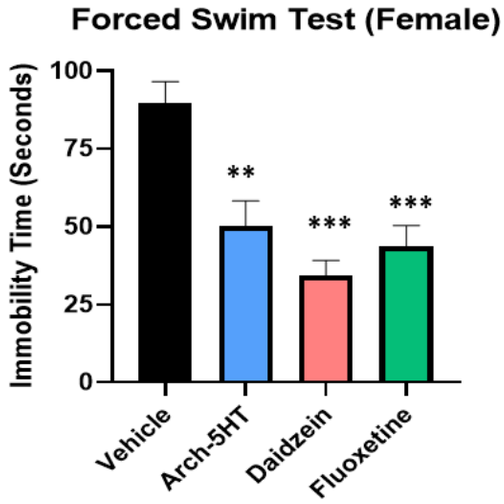

FIGURE 4. The bar graphs show the difference in immobility time in forced swim test among vehicle, daidzein, Arch-5HT and fluoxetine treated groups. (a) Forced swim test results of Male mice group (b) Forced swim test of Female mice group. Data are shown as mean ± SEM, with n=8, one-way ANOVA was used for statistical analysis. Daidzein (20mg/kg) has significantly decreased the immobility time as compared to vehicle treated group. **p<0.01; ***p<0.001 vs. vehicle treated group

Figure 5(a):

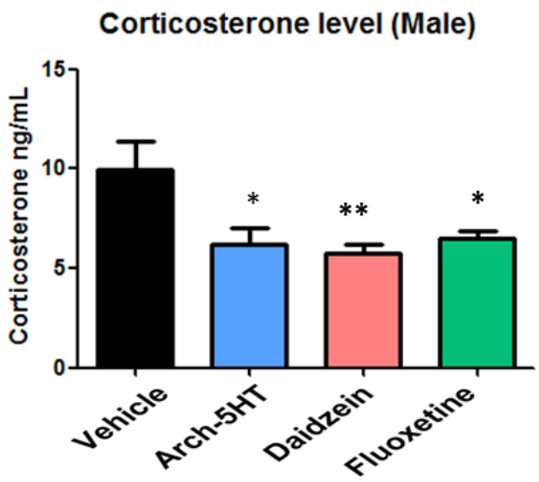

Figure 5(b):

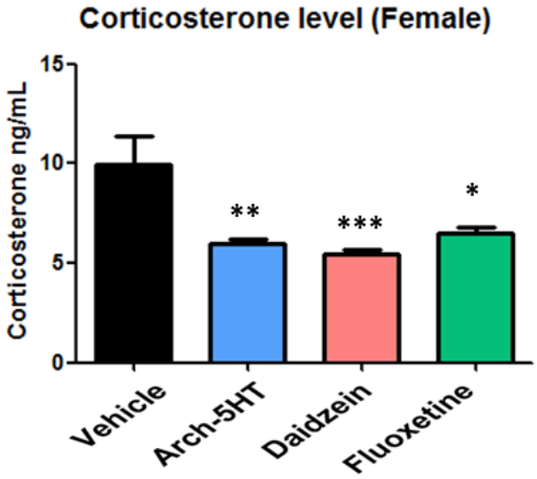

FIGURE 5. The bar graphs show the difference in plasma corticosterone levels of vehicle, daidzein treated, Arch-5HT and fluoxetine groups after 14 days of i.p treatment (a) Plasma corticosterone levels of Male mice group, and (b) Plasma corticosterone levels of Female mice groups. Data are shown as mean ± SEM, the number of mice in each group is 8, one-way ANOVA was used for statistical analysis. *p<0.05; **p<0.01; ***p<0.00 vs. vehicle treated group

DISCUSSION

There are preclinical and clinical evidence that suggest the role of endocannabinoid system in depression (Chadwick et al. 2020). The findings indicated that Daidzein, which is a natural compound, has promising pharmacological activities by modulating endocannabinoid system involved in depression. FAAH is the key enzyme of endocannabinoid system which is responsible for the hydrolysis of a number of fatty acid amide class signaling lipids, and anandamide is one of them (Cravatt et al. 1996). FAAH is an emerging target with promising therapeutic potential for many neurological disorders (Ren et al. 2020) such as Alzheimer's disease (Montanari et al. 2016) and post-traumatic stress disorder (Ahmad et al. 2020).

Molecular docking provides valuable sources to study the interaction of different compounds with biological targets such as receptors and enzymes (Myllymäki et al. 2009). Computer-assisted approaches are commonly used in current medicinal chemistry to advance the productivity of the discovery phase as well as to elaborate the structure activity relationship from natural and synthetic compounds. FAAH has four vital binding positions. Firstly, Ser241, Ser217, and Lys142 catalytic triad is responsible for the enzymatic hydrolytic activity. The OH group of Ser241 has the capability to catalytically attack on the carbonyl carbon of the FAAH. Ser-Ser-Lys triad is accountable for the capability of FAAH to break down lipid signaling molecule (McKinney & Cravatt 2003). Secondly, Gly240, Gly239, Ser241, and Ile238 provides another substrate for binding. Thirdly, an active door that is opened by Phe432 and Trp53 leads the substrate via catalysis. Lastly, Phe381 and Asp403 form an entrance for the substrate (Jaiswal et al. 2018). FAAH has an exceptional catalytic mechanism among other mammalian enzymes that comprise a catalytic triad with two serine residues (Ser217 and Ser241) and one lysine residue (Lys142), whereas the other share serine–histidine–aspartate triad is found in classical serine hydrolases enzymes (McKinney & Cravatt 2005). Hence, these serine residues provide the most suitable binding site for compounds. The computational data showed that Daidzein bounds to FAAH at the catalytic triad. Daidzein (OH-group) bounds to Ser-241 to inhibit the activity of FAAH. Therefore, it may catalytically attack the carbonyl carbon of fatty acid amide. The results of rigid-receptor docking replication demonstrated that Daidzein is a probable FAAH inhibitor with binding free energy which is higher than PF-750 but lower than Arch-5HT. Previously, Thors et al. (2007) reported that the uptake of anandamide was inhibited by Daidzein and the selective inhibition of FAAH was reported in various cell lines that expressed different levels of FAAH.

in-vitro data of daidzein inhibition has shown promising results. Daidzein has shown FAAH inhibition in a dose-dependent manner. Previously, flavonoids were shown to be useful in treating anxiety and stress through augmentation of ECS by inhibiting FAAH activity (Ahmad et al. 2020). Thors et al. (2010) conducted a study that showed the effect of biochanin-A, daidzein, genistein and formononetin on animal model of pain by inhibiting FAAH. However, the study suggested the antidepressant effect of Daidzein by potentially targeting the FAAH, although this concept was not proven through the animal study with the present experimental design.

Behavioural tests are frequently used to study the effect of compounds on neuropsychiatric diseases that include depression. Open field test is the most recommended test to study the exploratory and locomotor activity. In the current study, Daidzein showed no significant difference than that of fluoxetine and Arch-5HT treated groups in the open field test. However, there was an increasing trend of locomotion in Daidzein and Fluoxetine treated groups, therefore depicting decrease in depression. Previous study reported significant increase in locomotor activity after undergoing Daidzein treatment in Balb/c mice model (Zeng et al. 2010). Earlier studies showed that FAAH inhibition leads to increased mobility among rodents (Seillier et al. 2014). There was no difference in locomotor activity among the male and female groups. The meta-analysis of 293 articles concluded that the estrous cycle of female mice was not needed to be mentioned when utilizing female mice in scientific research because there was no significant difference in molecular and behavioural trail among male and female mice (Prendergast et al. 2014).

Several behavioural paradigms such as forced swim test, chronic mild stress, tail suspension test and learned helplessness test are used to study the depression-like behaviour in rodents. In the present study, the forced swim test was performed to evaluate anti-depressant activity of daidzein (Chen et al. 2021). Daidzein demonstrated decreased level of depression by showing less time of immobility in the forced swim test. Fluoxetine and Arch-5HT treated groups showed a decrease in immobility time, but Daidzein had more significant effect. These results were consistent with previously reported results, which showed that the Daidzein treatment improved the symptoms of chronic depression in the forced swim test (Sun & Qian 2011). A study also reported that enhancing the CB1 receptor signaling results in anti-depressant effect in forced swim test. Hence, it was suggested that the FAAH inhibitory activity of Daidzein leads to the anti-depressant effect and increased motility in behavioural tests. Chen et al. (2021) reported the antidepressant-like effect of Daidzein by attenuating HPA axis, decreasing levels of inflammatory cytokines and stress hormones on chronic mild stress as well as learned helplessness model in rats at a dose of 20 mg/kg/qd for 3 weeks.

Preclinical data suggested the role of endocannabinoid system in regulating the hypothalamus-pituitary-adrenal (HPA) axis (Bedse et al. 2014). The HPA axis is a neuroendocrine stress response system in mammals and has a key role in the pathogenesis of depression and anxiety-like disorders (Micale et al. 2007).

This HPA axis modulates the release of corticosterone and controls stress and depression through releasing these hormones. Depression leads to neuronal signals that are directed to hypothalamus. This triggers the release of vasopressin and corticotrophin releasing hormone. These two hormones couple together to stimulate the master pituitary gland. Hence, there is an enhanced synthesis and release of adrenocorticotropic hormone in blood by pituitary gland (Cota et al. 2007). This adrenocorticotropic hormone binds with specific receptors on adrenal gland and elevates the level of cortisol/corticosterone (Micale & Drago 2018). The activated HPA axis increases corticosterone levels, thus resulting in the alteration in synthesis and release of endocannabinoid molecules. The prolonged corticosterone levels also affect the expression of cannabinoid receptors (Morgese et al. 2017; Steiner & Wotjak 2008). The findings in this study also showed similar results. After conducting forced swim tests, the vehicle treated group showed increased levels of corticosterone. Daidzein decreased the plasma corticosterone levels to the same extent as shown by Arch-5HT (the endogenous FAAH inhibitor) and fluoxetine. The effect of Daidzein on female group was more pronounced than the male group. Krebs-Kraft et al. (2010) reported that female cortex has higher levels of anadamide degrading enzymes FAAH and monoacylglycerol lipase than male cortex region. This could be the reason behind the higher efficacy of isoflavonoids in female mice as compared to male mice. Therefore, it could be concluded that the Daidzein produces its antidepressant effect by inhibiting FAAH enzymes and ultimately regulating corticosterone levels.

In this study, different paradigms were used to elucidate the antidepressant effect of Daidzein. Daidzein has shown a prominent result as FAAH inhibitor in *in-silico* analysis and *in-vitro* studies. This natural flavonoid has likewise shown to treat depression by regulating the corticosterone levels that is associated with HPA axis. Behavioural paradigm also reported that Daidzein and fluoxetine exhibit the same way of treating the mice model. Fluoxetine which is a selective serotonin reuptake inhibitor has effect on anadamide levels and may affect the FAAH activity, whereas serotonergic receptor activation releases the anandamides in synaptic cleft and activates CB1 receptors. This behavioural effect of Daidzein might be due to direct effect of FAAH inhibitory (as shown by *in-vitro* and *in-silica* analysis) or indirect effect of serotonergic receptor activation that ultimately released anandamides, hence modulating endocannabinoid system. However, further investigation of Daidzein on anandamide levels in brain and cannabinoid receptor agonist/antagonist activity might provide a strong basis on how to use Daidzein as a therapeutic agent.

Conclusion

To summarize the findings, this study demonstrated that Daidzein can be used as a therapeutic agent to treat depression potentially by inhibiting the FAAH enzyme and targeting the endocannabinoid system. The FAAH inhibition might be the underlying mechanism involved in improving depression-like behaviour in mice model and decreasing plasma corticosterone levels. However, further studies are needed to be conducted. This study contributes to the growing literature of different compounds that may use FAAH inhibitor to combat neurological illness.

Supplementary Data

Pre-testing of three different doses of Daidzein were carried out with 10, 20, and 30 mg/kg on male and female mice. After 14 days of treatment, forced swim test was performed. The inconsistent results were obtained at doses of 10 and 30 mg/kg. Hence, 20 mg/kg dose was selected for *in-vivo* testing (Supplementary Figure S1).

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Figure S1(a):

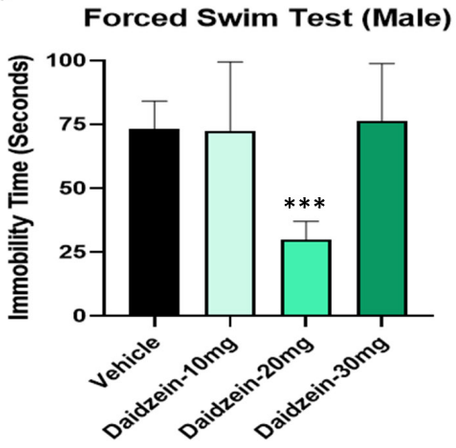

Figure S1(b):

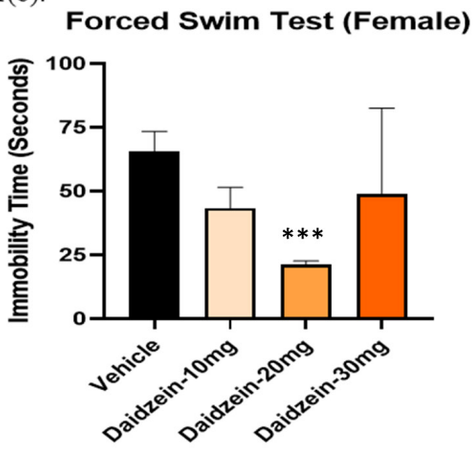

FIGURE S1. The bar graphs show the difference in immobility time in forced swim test among vehicle, Daidzein 10 mg/kg, Daidzein 20 mg/kg, Daidzein 30 mg/kg i.p treated mice groups. (a) Forced swim test results of Male mice group (b) Forced swim test of Female mice group. Data are shown as mean ± SEM, n = 8, ***p<0.001