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EDITORIAL

Mammalian target of rapamycin; novel insight for management of inflammatory bowel diseases

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Abstract

Inflammatory bowel diseases (IBDs), with blurred etiology, show a rising trend and are of global concern. Of various factors involved in IBD pathogenesis and development, inflammation has been shown to play a major role. Recognition of the molecular and cellular pathways that induce IBD is an emerging subject to develop targeted therapies. Mammalian target of rapamycin (mTOR) is one the most common receptors of many inflammatory pathways, including that of IBD. To this end, we intend to overview the mTOR inhibitors for their possible efficacy in present and future approaches to treatment of IBD.

Key Words: Inflammatory bowel diseases; Inflammation; Mammalian target of rapamycin; Mammalian target of rapamycin inhibitors

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Core tip: Inflammation is the main participant in the pathogenesis and development of inflammatory bowel disease (IBD). Since the mammalian target of rapamycin (mTOR) pathways are suggested to be involved in IBD progression, inhibition of the mTOR signaling may lead to a novel treatment modality for patients with IBD. Several biologics and synthetic and natural compounds have been introduced as mTOR inhibitors, which may control or eradicate intestinal inflammatory conditions such as IBD.

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INTRODUCTION

Inflammatory bowel diseases (IBDs) include two major types: ulcerative colitis (UC) and Crohn's disease (CD) that mainly progress due to abnormal dysregulation of the immune system[1]. Aberrations of the innate and adaptive immune responses and inflammatory processes play crucial roles in IBD pathogenesis[2]. Unregulated immune response stimulates Toll-like receptor-4 and the gastrointestinal enteric bacteria flora, thus activating the mucosal T cells and interferon (IFN) production and release. These events initiate the signal transduction cascades such as the nuclear factor (NF)-κB pathway; the mammalian target of rapamycin (mTOR) and transducer and activator of transcription 1 (STAT1) pathway. As a result, the activation of these pathways leads to elevation of inflammatory cytokines and induction of IBD. In the next step, leukocytes are aberrantly activated, leading to enhanced infiltration into the injured colonic site.

Therefore, modulation of inflammatory cytokines and chemokines could be important for IBD treatment. Pharmacological products or surgery are commonly used in IBD patients. Anti-inflammatory drugs (*i.e.* corticosteroids and aminosalicylates); immunomodulatory treatments (*i.e.* azathioprine, mercaptopurine, cyclosporine and methotrexate); biologic compounds [*i.e.* tumor necrosis factor (TNF)-α inhibitors]; and antimicrobials (*i.e.* ciprofloxacin and metronidazole) are current therapeutic options for IBD treatment. Among them, mTOR is a serine/threonine protein kinase of the phosphatidylinositol-3 kinase related kinase (PIKK) family, and a critical regulator of the inflammatory pathways[3,4]. mTOR has two subtypes of mTORC1 and mTORC2. Structurally, mTORC1 contains SEC13 protein 8 (mLST8)/G-protein b subunit-like protein (GbL), the regulatory-associated protein of mTOR (Raptor), DEPTOR, PRAS40, and a scaffold protein TTI1/TEL2 complex. The composition of mTOR, the mammalian stress-activated protein kinase interacting protein 1 (mSIN1), Tor2 (mTOR ortholog), mLST8/G_L, insensitive drug companion of mTOR (DICTOR), DEPTOR, TTI1, and TEL2 forms the mTORC2[5]. Activation of the mTOR pathway in intestinal epithelial cells has been shown to induce inflammation (Figure 1). In addition, mTOR is a downstream molecule of the PI3K/AKT/mTOR signaling pathway that plays a key role in the cellular transduction system and biological processes. Activation of the mTOR signaling pathway in the intestinal epithelial cells induces inflammation events that lead to IBD. In addition, activation of the mTOR/NF-κB pathway results in upregulation of IFN-γ, interleukin (IL)-6, IL-8, IL-1, and TNF-α. Induction of the P13K/AKT/mTOR pathway promotes TNF-α, IL-1β, transforming growth factor (TGF)-β, IL-12 and IL-6 secretion. The TLR/P38MAPK/mTOR pathway increases the serum levels of IL-12, IL-6, IL-8 and TNF-α. All of these pathways together could trigger IBD due to the induction of inflammatory processes. Activation of the mTOR signaling pathway induces immune cells such as macrophages and T cells, which in turn elevates the secretion of IFN-γ, IL-6, IL-8, IL-1 and TNF-α. It has been shown that regulatory T cells (Tregs) improve colitis through immunosuppression and reduction of inflammatory factors such as IL-1β, TNF-α, IL-6, IL-10 and IL-17A[6].

The myeloid-derived suppressor cells (MDSCs) are also attributed to the etiology of IBD and have been shown to improve colitis *in vivo*. It was demonstrated that mTOR inhibitors could suppress MDSCs and improve IBD. MDSCs have shown superior

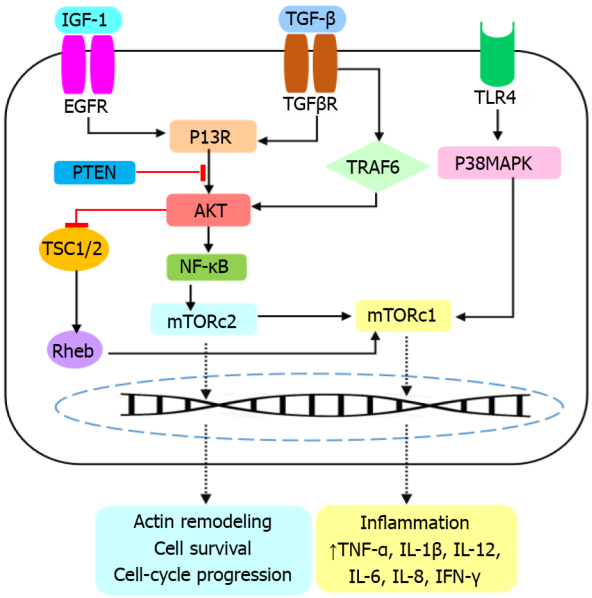

Figure 1 Activation of the mammalian target of rapamycin pathway in intestinal epithelial cells has been shown to induce inflammation. TNF: Tumor necrosis factor; IL: Interleukin; IFN: Interferon; IGF: Insulin-like growth factor; EGFR: Epidermal growth factor receptor; mTOR: Mammalian target of rapamycin.

immunosuppressive activities against IBD. mTOR inhibitors increase Tregs but reduce Th1 cells in IBD. These results indicate that some of the mTOR inhibitors attenuate IBD *via* Treg expansion promoted by MDSCs[6,7]. Inhibition of the mTOR pathway can improve IBD due to suppression of inflammatory processes. Hence, the factors that target the components of this pathway or the mTOR signaling proteins are of interest for drug development. Severe IBD could lead to several dangerous diseases such as colon cancer, irritable bowel syndrome, visceral hypersensitivity, neurodegenerative disease, *etc.* Induction of proinflammatory and inflammatory cytokines, *i.e.*, the cytokine storm in conditions such as COVID-19 may affect IBD patients. Therefore, the mTOR inhibitors are important not only to improve IBD, but also to reduce the risk of health-threatening conditions[8,9].

CONCLUSION

Given the high risk of inflammatory diseases and their influence on organ failure, new therapeutic triggers with fewer side effects and more specialization are needed. Clinical evidence demonstrates that inflammatory processes can increase the risk of many diseases. For example, it has been shown that inflammatory factors cause neurodegeneration and increase the risk of neurodegenerative disease such as Alzheimer's disease, Parkinson's disease and multiple sclerosis[10,11]. IBD could also lead to other gastrointestinal impairments such as colonic cancer. Recently, it has been proposed that IBD induces colonic angiotensin-converting enzyme 2 expression, probably due to the stimulation of cytokines storm, which finally increases susceptibility to COVID-19 and could end in death[12,13]. Although the etiopathogenesis of IBD is poorly understood, available evidence suggests that genetic susceptibility and environmental stimuli can predispose to immunological responses, and provoke IBD [14,15]. Many inflammatory signaling pathways participate in pathogenesis of IBD [16, 17]. The severity of IBD relies on the location and extent of the lesions, resulting in numerous extraintestinal manifestations. The mTOR signaling pathway is one of the most important mechanisms that contributes to progression of IBD. In this context, mTOR induces the NF-κB pathway, which together participate in production of several inflammatory mediators such as IFN-γ, IL-6, IL-8, IL-1 and TNF-α[18]. The TGF-β/P13K/AKT/mTOR pathway upregulates TNF-α, IL-1β, TGF-β, IL-12 and IL-6 expression. The TLR/P38MAPK/mTOR interaction increases serum levels of IL-12, IL-6, IL-8 and TNF-α[3]. Induction of the TLR4, MAPK and NF-κB pathways stimulates autophagy by the regulation of mTOR, thus improving the gut inflammatory responses and IBD[19]. These all suggest that inhibition of mTOR and/or the mTOR-dependent downstream signaling pathways represent promising insight for IBD

treatment. To assess such a hypothesis, several biologics such as everolimus, temsirolimus, deforolimus, sunitinib, bevacizumab, vedolizumab, etrolizumab, and the diverse mTOR analogs, AZD-8055, WYE-354, VS-5584, LY3023414, Ku-0063794, PI-103 and SKLB-M8 were analyzed and found to target mTOR and o block inflammatory processes[20,21]. Several natural compounds such as resveratrol, curcumin, acacetin, capsaicin, epigallocatechin-3, fisetin, harmine, panduratin A, prodigiosin, sinomenine, honokiol and isoliquiritigenin have shown the ability to inhibit mTOR. Taken together, targeting the mTOR signaling pathway could block secretion of cytokines and chemokines and not only improves IBD but also prevents the risk of other diseases, in which inflammation plays a key pathogenic role[6,22]. Future attempts should focus on planning clinical trials to evaluate the therapeutic efficacy of the mTOR inhibitors against IBD. Probable interaction of mTOR signaling with other pathways and effectors of IBD should also be considered to design targeted inhibitors with a broader action.

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6 Botanical, chemical, and pharmacological characteristics of *Lomatogonium rotatum*: A review

Dai LL, Eni RG, Fu MH, Ba GN

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Botanical, chemical, and pharmacological characteristics of *Lomatogonium rotatum*: A review

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Abstract

Lomatogonium rotatum (*L. rotatum*) Fries ex Nym, a dry whole grass belonging to the family Gentianaceae, is widely used to treat liver diseases in Mongolian medicine. In Mongolian medicine, *L. rotatum* Fries ex Nym, also known as *Digeda*, is a rare medicinal herb with low yield and widespread clinical use. Currently, it is included in over 25 traditional Mongolian medicine prescriptions that help reduce heat, dispel *xieri*, enhance stomach function, and heal wounds. Recent studies have shown that *L. rotatum* Fries ex Nym contains a variety of metabolites, including flavonoids, xanthone compounds, terpenoids, organic acids, steroids, and alkaloids. In addition, its anti-hepatitis B, anti-inflammatory, anti-acute liver injury, and anti-obesity effects have been proven by pharmacological studies. In this review, we summarize the ecological resources, traditional pharmacodynamics, chemical constituents, and pharmacological actions of *L. rotatum* Fries ex Nym, with an aim to provide a theoretical basis for future applied research and new product development.

Key Words: Mongolian medicine; *Lomatogonium rotatum*; Chemical composition; Pharmacological action; Research progress

Core Tip: As a highly distinctive Mongolian medicinal herb, *Lomatogonium rotatum* (*L. rotatum*) Fries ex Nym is traditionally used to prevent and treat liver and gallbladder diseases. However, its clinical application value and further development are limited by strict requirements on its growing environment, high demand for medicinal materials, decrease in wild resources, and insufficient scientific and technological availability in ethnic minority areas. Currently, a total of 38 compounds have been isolated and identified from *L. rotatum* Fries ex Nym, with flavonoids, xanthones, and terpenoids being the main metabolites. Pharmacological studies have mainly focused on hepatitis, liver injury, and weight loss, but mechanisms of pharmacological activity remain elusive and further comprehensive *in vivo* and *in vivo* experimental designs are needed to elucidate these issues.

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INTRODUCTION

Lomatogonium rotatum (*L. rotatum*) Fries ex Nym is a dry whole grass belonging to the family Gentianaceae. *L. rotatum* Fries ex Nym, also known as *Habirigen-Digeda* or *Temuri-Digeda*, is a commonly used Mongolian medicine to treat liver diseases. In traditional Mongolian medicine, *L. rotatum* Fries ex Nym is believed to clear heat, remove *xieri*, strengthen the stomach, treat poisoning, and heal wounds. It is widely used to prevent and treat influenza and fever, hepatobiliary disease, typhoid fever, and jaundice. It also has a therapeutic effect on heatstroke symptoms[1,2]. In addition, it is clinically effective in the prevention and treatment of liver and gallbladder diseases. Recent chemical and pharmacological studies have shown that *L. rotatum* Fries ex Nym contains organic compounds (xanthones), swertiamarin, oleanolic acid, luteolin, and other metabolites that play a role in enhancing the liver and biliary tract functions[3].

MORPHOLOGY, RESOURCES, AND BREEDING

Floral morphology of L. rotatum Fries ex Nym

L. rotatum Fries ex Nym is a 30- to 50-cm-high annual herb with an erect stem, four prisms, and a few branches. The leaves are sessile and opposite, and the leaf blades are narrowly lanceolate, 1.5 to 3 cm long, 0.4 to 0.6 cm wide, and apically acute, with a wider base. It has five calyces, deep, lanceolate lobes, and an equally long corolla. The corolla has five deeply lobed segments; the lobes are spheric and obtuse, with a toothed tube on each side of the base (Figure 1). The plant usually grows on hillsides and wetlands at an altitude of 2500 m. After picking the grass in the autumn flowering season, adherent soil and moisture are removed, and it is mashed or sun-dried before use.

Resources and geographical distribution of L. rotatum Fries ex Nym

L. rotatum Fries ex Nym for medicinal use primarily grows in the wild. However, the yield is low because of environmental factors and excessive mining, necessitating artificial breeding, which also has a low yield. A survey found wild *L. rotatum* Fries ex Nym in Xilingol League, Horqin, and Hulun Buir of the Inner Mongolia Autonomous Region, but observed a decreasing growth trend year on year[4]. The shortage of *L. rotatum* Fries ex Nym has led to the use of *Viola yedoensis* Makino of the family Violaceae as a substitute medicine in most Mongolian hospitals. It is, therefore, crucial to immediately protect and direct increasing efforts toward improving resources required for growing *L. rotatum* Fries ex Nym. Currently, several challenges, such as the low germination rate of group embryos and the low success rate of inoculation, which affect the large-scale cultivation of *L. rotatum* Fries ex Nym, continue to persist in artificial cultivation and domestication of the plant[4]. Li[4] conducted a field investigation and found that the plant shows optimum growth during the dry season in February, with an average temperature of -13 to 18 °C and precipitation of 6-19 mm. The vegetation growth types include temperate grasses, mixed grasses meadow, halophytic meadow, temperate deciduous shrub, and broad-leaved forest[5]. According to the literature, *L. rotatum* Fries ex Nym is widely distributed in Inner Mongolia, Gansu, Yunnan, Xinjiang, Qinghai, Tibet, Sichuan, and other regions[6], usually on grassy slopes of hillsides and shrublands below an altitude of 4200 m[7]. Li[4] pointed out that *L. rotatum* Fries ex Nym is scattered in shrublands, alpine meadows, grassland wetlands, flat meadow grasslands associated with rivers, and alpine and hillside meadows in Xilingol, Inner Mongolia, Heilongjiang, Hebei, and other regions[4].

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Figure 1 The whole plant of *Lomatogonium rotatum*.

Characteristics of breeding and pollen viability of L. rotatum Fries ex Nym

Li *et al*[7] set up fixed points in the field to monitor the morphology and characteristics of *L. rotatum* Fries ex Nym organs, dynamics during flowering, and types of pollinators. A systematic inspection and measurement of its growth and reproduction were conducted by calculating pollen viability, pollinable characteristics such as stigma, estimation of pollen-to-ovule ratio, and hybridization index, and artificially controlled pollination[7]. The results demonstrated that the flowering time of a single *L. rotatum* Fries ex Nym flower was 6-7 d and that the flowering time of a single plant could be classified into a common bud stage followed by the initial flowering, blooming, wilt, and litter periods. During this process, the open stigma was always higher than the anther, and the pollen vitality and stigma receptivity were relatively strong at 2-3 d after anthesis. In addition, the researchers also observed that the breeding system was mainly outcrossing, and some were self-compatible, which may require insect thrips as the primary pollinators. In the case of bagging without pollination after emasculation, the seed setting rate of the fruit was 0, indicating a lack of fusion reproduction[8]. Zhu *et al*[9] investigated the effects of different storage times and temperatures and the use of gibberellin reagents in promoting the flowering and growth rates of *L. rotatum* Fries ex Nym seeds. They found that changes in outdoor temperature and gibberellin immersion significantly promoted the germination and flowering of *L. rotatum* Fries ex Nym seeds[9].

Similarly, Li *et al*[10] intervened in the *L. rotatum* Fries ex Nym germination rate by adopting different temperatures and germination sites. They found that the highest germination index was on sand, under which condition the rot rate of the seeds was the lowest. Furthermore, the germination rate was highest at 40 °C. Therefore, the optimal conditions for *L. rotatum* Fries ex Nym seed germination may be the temperature of 40 °C and planting on sand[10].

TRADITIONAL APPLICATIONS

L. rotatum Fries ex Nym is a typical medicinal plant used for internal applications to prevent and treat various liver and gallbladder diseases. In 1998, it was recorded in the Drug Standard (Mongolian Medicine Volume) of the Ministry of Health. The plant helps degrade *xieri* and clears heat. In Mongolian medicinal prescriptions, *L. rotatum* Fries ex Nym is either combined with *Herpetospermum caudigerum* Wall and *Ixeris Chinensis* (Thunb.) Nakai to formulate a Lidan powder containing 28 medicinal herbs, or it is used alone to degrade the *xieri* heat of the gallbladder. In addition, it is used as a prescription combination of Digeda-15, Digeda-20, and Digeda-25 to treat common clinical diseases such as liver and gallbladder heat, redness, yellow appearance of the eye and skin, gallbladder stasis, and stasis of *xieri*, which may lead to organ injury[11]. In addition, Digeda-4, a combination of *L. rotatum* Fries ex Nym and *Coptidis rhizoma*, *Gardenia jasminoides* Ellis, and *Dianthus superbus* L., is used to reduce problems such as inflammation, sore throat, liver and gallbladder heat, blood heat, thirst, and irritability[2]. Furthermore, *L. rotatum* Fries ex Nym is used as an adjuvant, subordinate, or auxiliary medicine in several compound preparations. There are a total of 24 Mongolian medicine prescriptions that include *L. rotatum* Fries ex Nym, including three where *L. rotatum* Fries ex Nym is used as a monarch medicine, four where *L. rotatum* Fries ex Nym is used as a minister medicine, 16 as assistant medicines, and one as a guide medicine.

CHEMICAL COMPOSITION

Research on the pharmacodynamic substances and chemical components of *L. rotatum* Fries ex Nym is still in the initial stages. To date, 38 compounds have been isolated and identified, mainly including flavonoids and xanthones, with a small number of iridoids, alkaloids, steroids, organic acids, amongst others.

Flavonoids

Flavonoids are abundant in most herbaceous plants, especially in higher plants, and possess a plethora of biological activities. According to the literature, *L. rotatum* Fries ex Nym contains about 16 flavonoids, including luteolin[12], apigenin, 5,7,3',4',5'-pentahydroxy flavonoids, quercetin, kaempferol, luteolin-7-O-glucoside, apigenin-7-O-glucoside[13], swertisin[14], swertianolin[15], isoorientin, mangiferin, isovitexin[16], carinoside A[17], carinoside B, carinoside C, and carinoside D[18] (Table 1 and Figure 2).

Xanthones

Ten xanthone compounds have been identified in *L. rotatum* Fries ex Nym: 6,8-dihydroxy-1,2-dimethoxy xanthone[19], 1,8-dihydroxy-3,4,5-trimethoxyxanthone, 1-hydroxy-3,7,8-trimethoxy xanthone, 8-hydroxy-1,3,5-trimethyl xanthone, 1-hydroxy-3,5,8-trimethoxy xanthone[13], 1,8-dihydroxy-4,5-dimethoxy-6,7-methylenedioxy xanthone, 5-O-D-gluco-pyranosyl-1,3,8-trihydroxy-5,6,7,8-tetrahydroxanthone, 1,3,5,8-tetrahydroxy-5,6,7,8-tetrah-ydroxanthone[20], 1,2,6-trihydroxyl xanthone-8-O-β-D-glucoside, and 1,4,8-trimethoxyxanthone--6-O-β-D-glucoronyl-(1→6)O-β-D-glucoside[21] (Table 1 and Figure 2).

Terpenoids

Terpenoids are a class of compounds derived from methylcarboxylic acid with ≥ 2 isoprene units in the basic carbon frame. According to the literature, three iridoids (*i.e.*, swertiamarin[22-25], ursolic acid 3β-hydroxy-ursol-11,12-ene-28,13β-lactone[14], and amarogentin[16,26]), as well as two pentacyclic triterpenes, oleanolic acid[20], 2a-hydroxyoleanolic acid[13], lomacarinoside A, and lomacarinoside B [27] are present in *L. rotatum* Fries ex Nym (Table 1 and Figure 2).

Other compounds

L. rotatum Fries ex Nym also contains organic acids, steroids[14], alkaloids[28,29], and other compounds (erythrocentaurin), in addition to the above-mentioned metabolites[13]. However, current research on this aspect is inadequate, warranting further studies (Table 1 and Figure 2).

Study on extraction process of L. rotatum Fries ex Nym

Chen *et al*[30] investigated the primary factors affecting total flavonoid extraction in *L. rotatum* Fries ex Nym using single-factor experiments. They then optimized the extraction method for the total flavonoids in *L. rotatum* Fries ex Nym by an orthogonal test using the rutin concentration as the standard and formulated the optimal extraction process for the total flavonoids. The results demonstrated that the optimal parameters for total flavonoid extraction from *L. rotatum* Fries ex Nym were: Ethanol concentration, 60%; solvent volume, 150 mL; extraction time, 8 h; total flavonoid extraction rate from *L. rotatum* Fries ex Nym, about 3.47%.

In addition, single-factor experiments were conducted to explore the effects of the ethanol percentage and volume used for extraction, the ultrasonic extraction time, and the liquid-to-sample ratio on the total saponin concentration extracted from *L. rotatum* Fries ex Nym. Response surface methodology was used to optimize the experimental conditions for ultrasonic extraction of *L. rotatum* Fries ex Nym, resulting in a gradual increase in the total saponin extraction yield. The experimental results demonstrated that the optimal experimental conditions for the extraction of total saponins from *L. rotatum* Fries ex Nym were as follows: Average volume content fraction of ethanol, 77%; average liquid-to-solid ratio, 40 mL/g; ultrasound duration, 33 min. Under these conditions, the researchers found that the average total saponin concentration extracted from *L. rotatum* Fries ex Nym was 27.36 mg/g, which was close to the theoretically predicted value[31]. However, the optimization of the extraction process, using 65% ethanol, a solid-to-liquid ratio of 20 mL/g, and an extraction time of 20 min, defined by Liu *et al*[32]'s study, is considered the best method[32].

Fingerprint and mineral elements of L. rotatum Fries ex Nym

Sun *et al*[33] used high-performance liquid chromatography to determine the fingerprint of *L. rotatum* Fries ex Nym plants from 15 different places and cultivation areas. The results showed 15 common peaks, of which the five most common were swertiamarin, isoorientin, swertisin, apigenin, and luteolin. In addition, Yuan *et al*[34] measured the mineral elements in two *Lomatogonium* species using inductively coupled plasma-optical emission spectrometry, finding 21 mineral elements in *Lomatogonium macranthum* and 18 elements in *Lomatogonium carinthiacum*. Both *Lomatogonium* species had relatively high Ca, Mg, and Fe contents while the greatest difference was seen in the Co concentrations and the least difference was found in the Ti contents[34].

Table 1 Chemical composition of *Lomatogonium rotatum*

No.	Compound	Ref.
Flavonoids		
1	Luteolin	[12]
2	Apigenin	[13]
3	5,7,3′,4′,5′-pentahydroxy flavonoids	[13]
4	Quercetin	[13]
5	Kaempferol	[13]
6	Luteolin-7-O-glucoside	[13]
7	Apigenin-7-O-glucoside	[13]
8	Swertisin	[14]
9	Swertianolin	[15]
10	Isoorientin	[16]
11	Mangiferin	[16]
12	Isovitexin	[16]
13	Carinoside A	[17]
14	Carinoside B	[18]
15	Carinoside C	[18]
16	Carinoside D	[18]
Xanthones		
17	6, 8-dihydroxy-1, 2-dimethoxy xanthone	[19]
18	1-hydroxy-3,7, 8-trimethoxy xanthone	[13]
19	8-hydroxy-1,3, 5-trimethyl xanthone	[13]
20	1-hydroxy-3,5, 8-trimethoxy xanthone	[13]
21	1, 8-dihydroxy-3, 4, 5-trimethoxyxanthone	[13]
22	5-O-D-glucopyranosyl-1,3,8-trihydroxy-5,6,7,8-tetrahydroxanthone	[20]
23	1,3,5,8-tetrahydroxy-5,6,7,8-tetrah-ydroxanthone	[20]
24	1, 8-dihydroxy-4, 5-dimethoxy-6, 7-methylene dioxy xanthone	[20]
25	1,2, 6-trihydroxyl xanthone-8-O–β-D-glucoside	[21]
26	1,4,8-trimethoxyxanthone-6-O–β-D-glucoronyl-(1→6)O-β-D-glucoside	[21]
Terpenoids		
27	Oleanolic acid	[20]
28	2a-hydroxyoleanolic acid	[13]
29	Swertiamarin	[22-25]
30	Amarogentin	[16,26]
31	Ursolic acid 3 β-hydroxy-ursol-11, 12-ene-28, 13β-lactone	[14]
32	Lomacarinoside A	[27]
33	Lomacarinoside B	[27]
Organic acids		
34	Ferulic acid	[12]
35	Caffeic acid	[29]
Alkaloids		
36	Berberine hydrochloride	[28]

Steroid		
37	Daucosterol	[14]
Other		
38	Erythrocentaurin	[13]

PHARMACOLOGICAL ACTION OF *L. ROTATUM* FRIES EX NYM

Effects of L. rotatum Fries ex Nym powder on hepatitis B

Bai *et al*[35] investigated the anti-hepatitis B effect of *L. rotatum* Fries ex Nym powder and found that *L. rotatum* Fries ex Nym had a relatively low cytotoxicity to HepG2 cells, with some inhibitory effect on the number of hepatitis B virus (HBV) DNA copies in the cells. In addition, *in vitro* experiments showed that *L. rotatum* Fries ex Nym is able to counteract HBV to some degree. This may be because *L. rotatum* Fries ex Nym acts against HBV by directly inducing apoptosis, thereby blocking HBV replication in HepG 2 cells.

Anti-inflammatory effects of L. rotatum Fries ex Nym

Ethyl acetate has been found to be the active component responsible for the anti-inflammatory effect of *L. rotatum* Fries ex Nym, which has significant antibacterial activity against both Gram-positive and -negative bacteria such as *Escherichia coli*, *Staphylococcus alba*, drug-resistant *Staphylococcus aureus*, *S. aureus*, and *Pseudomonas aeruginosa*[36]. *L. rotatum* Fries ex Nym also contains several anti-inflammatory compounds, including sweroside, swertiamarin, and luteolin. Chen *et al*[37] reported that swertiamarin had anti-inflammatory, antioxidant, and anti-fibrotic effects in rats with smoking-exposed prostate dysfunction[37]. Aziz *et al*[38] also reported that luteolin had strong anti-inflammatory effects in both *in vivo* and *in vitro* experiments[38].

Anti-acute liver injury effect

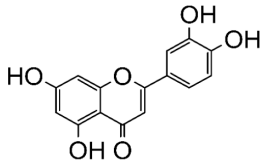
L. rotatum Fries ex Nym extracts with different polarities were reported to have pharmacological effects on acute liver injury[39]. In a drug interaction study of *L. rotatum* Fries ex Nym prescription medication, an *L. rotatum* Fries ex Nym water extract was found to significantly protect liver function in rats with carbon tetrachloride (CCl_4)-induced liver astrocyte damage[40]. In a further study, the urine of rats with liver injury was analyzed after *L. rotatum* Fries ex Nym administration, showing 19 common peaks, one of which was the drug itself, 14 were metabolites passed through the body, and the rest were endogenous components of urine[41]. In addition, the study found that the Digeda-4 decoction had a protective action in mice with pyloric ligation-induced liver injury. It has been suggested that *L. rotatum* Fries ex Nym may affect the protein expression of MRP3 and MRP4 by regulating the nuclear receptors CAR and PXR, resulting in liver protection[42]. An experimental metabolomics study found that *L. rotatum* Fries ex Nym administration restored several disturbed metabolic pathways, including those involving linoleic acid and glycerolipid metabolism. The use of another eight metabolites as potential biomarkers was proposed to help clarify the liver protective mechanism of *L. rotatum* Fries ex Nym[43]. Zhao *et al*[44] also proposed that the liver protective activity of *L. rotatum* Fries ex Nym may be related to metabolites in rat plasma and liver[44].

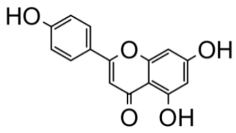
Anti-obesity effect

Bao *et al*[45] investigated the effect of *L. rotatum* Fries ex Nym on weight loss based on the function of bitter receptors in rats with obesity induced by a high-fat and high-energy diet. The *L. rotatum* Fries ex Nym extract significantly reduced body weight, Lee's index, epididymal fat, perirenal fat, and mesenteric fat deposition in the rats. It also reduced serum triglyceride and total cholesterol levels to a certain extent, indicating its potential for lipid-lowering, cholesterol-lowering, and weight-loss effects. Chemical analysis demonstrated that flavonoids, glycosides, and alkaloids were the primary components of *L. rotatum* Fries ex Nym, and the main source of bitterness was base substances. The effects of *L. rotatum* Fries ex Nym on fat metabolism and its bitter receptor activation mechanism require further investigation[46].

CONCLUSION

As a highly distinctive Mongolian medicinal herb, *L. rotatum* Fries ex Nym is traditionally used to prevent and treat liver and gallbladder diseases. However, its clinical application value and further development are limited by the strict requirements of its growing conditions, high demand for medicinal materials, decrease in natural resources, and insufficient scientific and technological expertise in ethnic minority areas. To date, a total of 38 compounds have been isolated and identified from *L.*


Luteolin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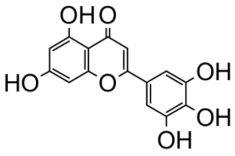
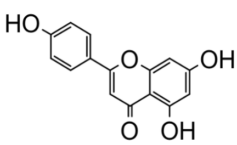

5, 7, 3', 4', 5'-pentahydroxy flavonoids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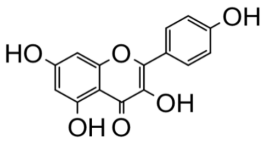
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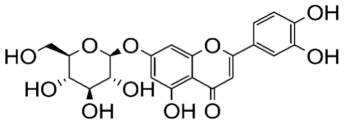
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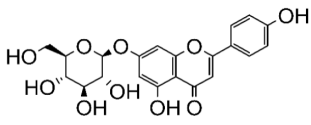
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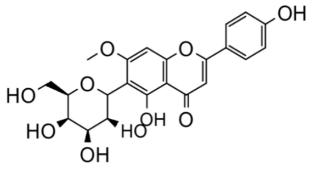
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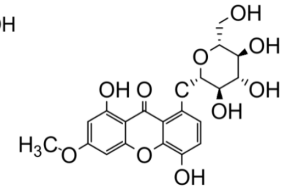
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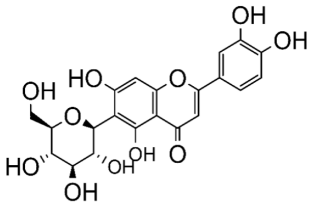
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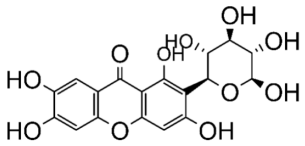
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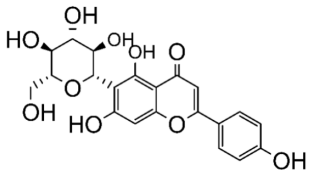
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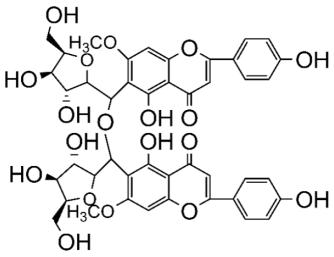
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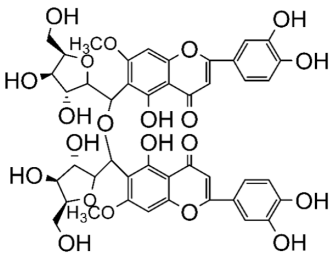
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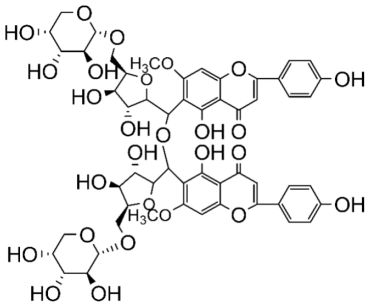
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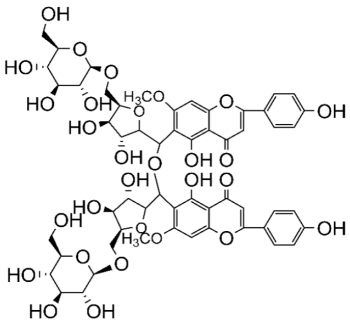
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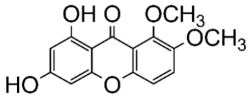
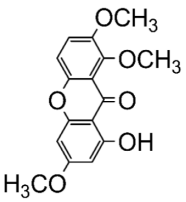

6, 8-dihydroxy-1, 2-dimethoxy xanthone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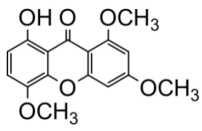
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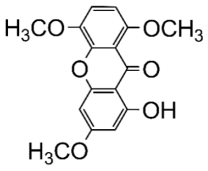
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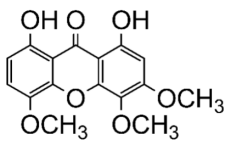
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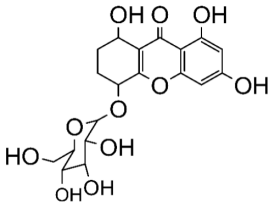
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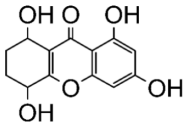
1, 3, 5, 8-tetrahydroxy-5, 6, 7, 8-tetrahydroxanthone

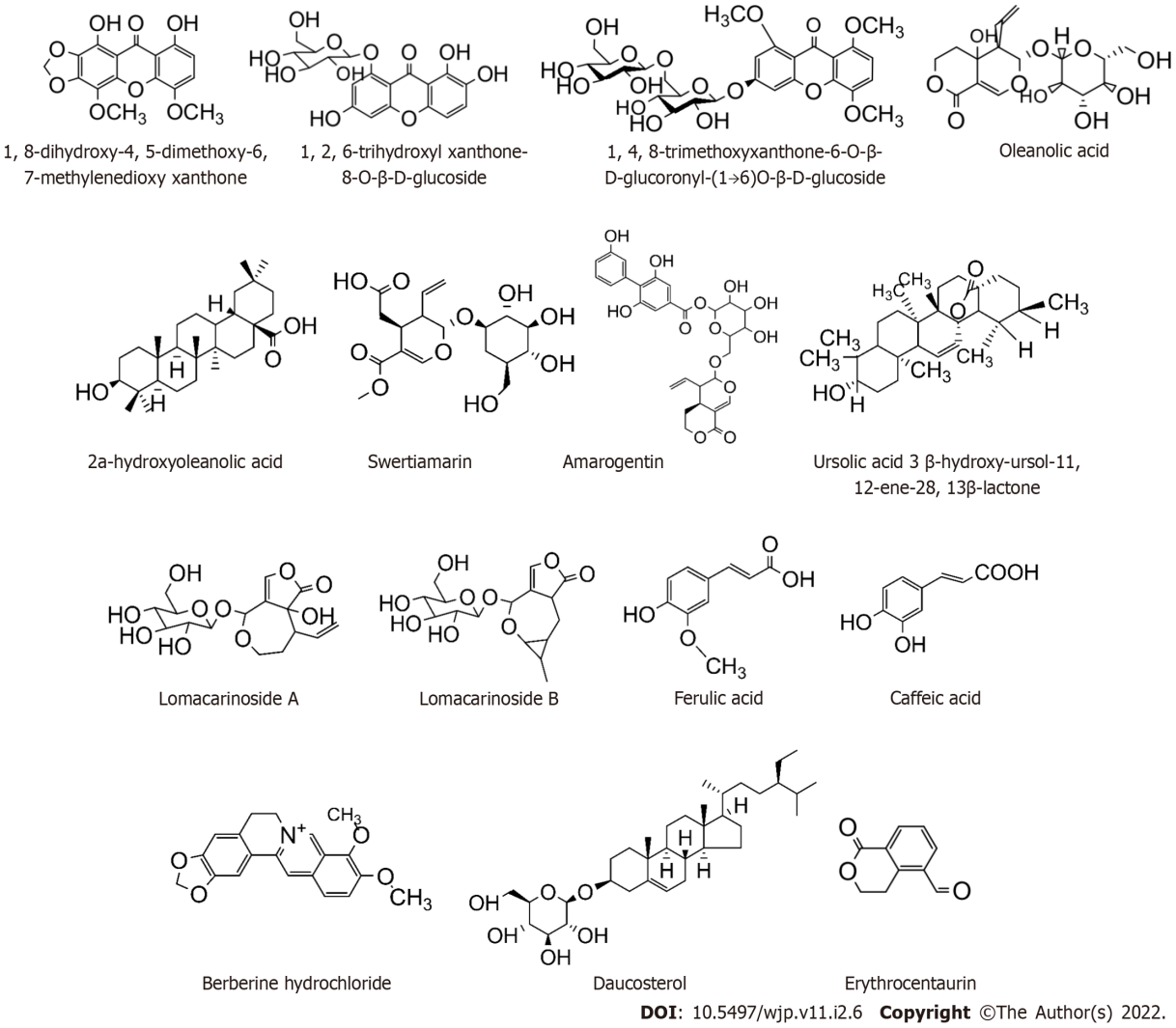

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Figure 2 Structure of compounds 1-38 from *Lomatogonium rotatum*.

rotatum Fries ex Nym, with flavonoids, xanthones, and terpenoids being the main metabolites. While pharmacological studies on *L. rotatum* Fries ex Nym have mainly focused on hepatitis, liver injury, and weight loss, the mechanisms of its pharmacological activity remain elusive and further comprehensive *in vivo* and *in vivo* experimental studies are necessary. Thus, our study may provide a foundation for further research on *L. rotatum* Fries ex Nym and its clinical applications.

FOOTNOTES

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REVIEW

Controversial usages of kratom (*Mitragyna speciosa*): For good or for evil

Murtadha Basheer, Rana Khudhair Jasim, Gam Lay Harn

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Abstract

Kratom (*Mitragyna speciosa*) is a plant that grows well in tropical climates such as in Southeast Asia. Traditionally, people discovered it possessed a stimulating effect that relieved tiredness. Furthermore, it contains analgesic and medicinal properties for the treatment of pain, diarrhea, muscle discomfort, and blood pressure and to enhance stamina. Nevertheless, long term or regular consumption of kratom leads to addiction. This is because the main alkaloid of kratom, mitragynine, binds to opioid receptors and exerts a euphoric effect similar to that of morphine, which may lead to death. Due to this reason, kratom has been listed as a regulated substance in many countries including the United States, Thailand, Malaysia, Bhutan, Finland, Lithuania, Denmark, Poland, Sweden, Australia, and Myanmar. Usages of kratom carry two pharmacological effects depending on dosage. Low-dose kratom exerts a stimulating effect that refreshes the user. High-dose kratom exerts sedative effects that can lead to addiction similar to that of morphine. Despite the euphoric effect of kratom, the beneficial values of kratom to human health is indisputable. Therefore, a complete banning of kratom may cause a loss to pharmaceutical industry. Rather, a controlled or selective usage of kratom will be a better choice.

Key Words: Kratom; Opioid; Pharmacological actions; Toxicity; Addiction; Herbal plant

Core Tip: Traditionally, people discovered kratom (*Mitragyna speciosa*) possessed a stimulating effect that relieved tiredness. Long term or regular consumption of kratom leads to addiction because the main alkaloid of kratom binds to opioid receptors and exerts a euphoric effect. Due to this reason, kratom has been listed as a regulated substance in many countries. Despite the euphoric effect of kratom, the beneficial values of kratom to human health is indisputable. Therefore, a complete banning of kratom may cause a loss to pharmaceutical industry. Rather, a controlled or selective usage of kratom will be a better choice.

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INTRODUCTION

Kratom (*Mitragyna speciosa*) (Figure 1) is a plant native to Southeast Asia. It has been planted as a recreational herb due to its analgesic properties[1]. Kratom was originally recorded for its stimulating effect. The leaves of the tree that are exploited for its pharmacological actions contain different colored veins (white, green, or red) that have been connected to a variety of effects[2]. The red vein leaf is popular in Thailand for its potency[3]. Traditionally, the raw leaves were chewed for their analgesic and soothing effect[2]. In addition, kratom leaves have been used to treat diarrhea, muscle discomfort, decrease blood pressure, and enhance stamina in Southeast Asia[4]. Antispasmodic, muscle relaxant, and antidiarrheal properties of kratom are still in use in the region, while its stimulant and analgesic effects are popular home remedies[3,5].

Folk medicine in Southeast Asia has recognized kratom as an herb[6] in the form of "herbal tea." Its use in the searing heat of the tropics helps workers stay alert and productive. Kratom is widely used to wean morphine addicts off the drug[7]. In fact kratom was utilized historically as an opioid substitute, and it was once widely used in Malaysia and Thailand as an opium replacement and countermeasure [8].

Nevertheless, kratom use has been banned by the local government in Malaysia[8], where it was classified as a poison under the Poison Act[6]. In Thailand, kratom was classified as a Schedule 5 substance under the Thai Narcotics Act. Bhutan, Finland, Lithuania, Denmark, Poland, Sweden, Australia, and Myanmar have kratom under control or regulation[6]. The United States also regulated the use of kratom when the United States Drug Enforcement Administration classified it as a drug of concern[9]. The Centers for Disease Control and Prevention (CDC) released a study on the harmful effects of kratom use on health, where 660 reports on the exposures were documented[10]. In addition, the CDC also documented hundreds of deaths connected with kratom usage[11,12]. Furthermore, the Food and Drug Administration does not acknowledge it as a recognized supplement. Subsequently, the prominence of kratom in the American psyche was reintroduced, where mitragynine and 7-hdroxymitragynine were announced as substances to be added to Schedule I of the Controlled Substances Act by the Drug Enforcement Administration. The Drug Enforcement Administration statement classified the chemicals as Schedule I, meaning kratom has no recognized medicinal value and a significant potential for misuse[13]. Despite all these regulations, several nations continue to allow kratom use today as there is no conclusive evidence that kratom use has the same negative health consequences as conventional opioids[14].

Although the Poisons Act of 1952 makes it illegal to consume kratom in Malaysia, the native tree and tea decoctions are abundantly available in the country[15]. In the United States, kratom products can be purchased from shops and online distributors. Kratom products are available in a variety of forms, including tablets, tea drinks, and powders[10,15]. Increased sales of kratom in Europe and North America have increased worries about its safety and prompted some European governments to prohibit the plant and its active alkaloids[16].

Kratom was legalized in Thailand in 2018 for therapeutic use after a prohibition on its usage, manufacture, and possession was overturned[17]. Following this legalization, many other countries may follow suit. In view of the potential negative effects of kratom, would this legalization be beneficial to society?

PHARMACOLOGICAL ACTIVE ALKALOIDS OF KRATOM

More than 40 compounds were isolated and chemically characterized from *Mitragyna speciosa* since the 1960s[18]. Thus far, only four of these components are pharmacologically active, namely mitragynine, 7-

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Figure 1 Kratom plant.

hydroxymitragynine, speciociliatine, and corynantheidine[19,20]. Mitragynine is the most common alkaloid of the kratom plant[21], and it can be easily oxidized[21]. Mitragynine makes up 66% of the alkaloid content of kratom. On the other hand, 7-hydroxymitragynine was identified as a minor ingredient of kratom leaves extract[6] that makes up 0.04% of the alkaloids[22]. Speciogynine, paynantheine, and mitraphylline are also indole alkaloids of kratom[23]. These compounds are not pharmacologically active, but they contribute synergistically to the overall effect of kratom that formed the diversity of alkaloids found in kratom extracts.

REPORTED MECHANISMS FOR PHARMACOLOGICAL EFFECTS OF KRATOM

Mitragynine and 7-hydroxymitragynine have the ability to target opioid receptors, yet their binding affinity to opioid receptors is significantly different[24]. Mitragynine has a lower binding affinity to opioid receptors than morphine, while 7-hydroxymitragynine is significantly more powerful than either, which is approximately 46 times the potency of mitragynine and 13 times the potency of morphine[25,26]. Therefore, 7-hydroxymitragynine has been targeted as the most important factor in the development of addiction and toxicity, while mitragynine poses a small danger[27,28]. The greater binding affinity of 7-hydroxymitragynine to opioid receptors is due to the addition of a hydroxyl group at the C7 position[19]. Both mitragynine and 7-hydroxymitragynine have been demonstrated to work as agonists, with mitragynine activating primarily μ- and δ-receptors and 7-hydroxymitragynine activating primarily μ- and κ-receptors[26,29,30]. Nonetheless, contradictory evidence suggests a different view. Rather than acting as simple agonists, mitragynine and 7-hydroxymitragynine appear to exert differential effects on distinct receptors[21] in which mitragynine and 7-hydroxymitragynine exert both agonistic and antagonistic characteristic upon binding to opioid receptors. On the other hand, they are partial agonists to μ-receptors, competitive antagonists to δ-receptors, and their effects on κ-receptors are very minimal[31].

Kratom contains indole alkaloids. These indole alkaloids are structurally and pharmacodynamically unlike its opioid rival. Therefore, they were identified as atypical opioids in order to distinguish them from morphine, semisynthetic opioids, and endogenous ligands[32]. Upon binding to opioid receptors, the indole alkaloids (such as kratom alkaloids) activate G-protein-coupled receptors. However, unlike conventional opioids (such as morphine), indole alkaloids do not initiate the β-arrestin pathway when they activate G-protein-coupled receptors[5]. This process refers to biased agonism or ligand-directed signaling that permits a single receptor to exert numerous distinct intracellular effects by selectively disabling the receptor's various signaling cascades[33]. It is worth noting that symptoms of opioid use like respiratory depression, sleepiness, and constipation are due to β-arrestin recruitment[34,35]. The selective β-arrestin inactivation by mitragynine is a desirable trait for an opioid. Therefore, mitragynine may serve as a useful template for the development of novel opioids with more tolerable side effects [21].

Apart from its opioid-like analgesic actions, mitragynine appears to inhibit pain signals *via* other pathways than morphine. Implying a multimodal involvement in pain perception regulation. For example, mitragynine bears a high degree of structural similarity to yohimbine, another indole alkaloid with well-documented adrenergic effects[24]. Due to this similarity, mitragynine analgesic properties

appear to act similarly as yohimbine, which is through activating the α-2 adrenergic postsynaptic receptors[36,37]. α-2 receptors are found in pain modulatory "descending" pathways. These pathways constitute a significant improvement in complicated neurobiological knowledge of pain[38,39]. Another study showed that mitragynine inhibits neuronal pain transmission *via* Ca^{2+} channel blockage[30]. When cellular connections are considered, the release of neurotransmitters was inhibited from the nerve terminals of the vas deferens[2] by the occlusion of neuronal Ca^{2+} channels[6,40].

The indirect analgesic qualities have been ascribed to anti-inflammatory activities of mitragynine, which are thought to be mediated through the suppression of *COX-2* and prostaglandin E2 mRNA expression[41,42]. Apart from these antinociceptive properties, mitragynine exhibits some affinity for D2 dopamine receptors, A2A adenosine receptors, and 5-HT2C and 5-HT7 serotonin receptors. All these belong to central nervous system receptors. Although the physiological significance of these interactions is unknown[30], postsynaptic α-2 adrenergic receptor stimulation and serotonergic 5-HT2A receptor blockage were reported to cause stimulant action of the central nervous system[43,44].

G-protein-biased signaling mechanism of action of mitragynine and 7-hydroxymitragynine makes kratom act as a partial agonist in terms of respiratory depressant effects[31,45,46]. The physiological impact of kratom is a combination of stimulant and sedative, depending on the dose. Stimulant effects are predominant at low dosages, while sedative effects are predominant at higher dosages[15,47]. This differential effect is due to the assortment of alkaloids shown in kratom extricates, which is a distinctive potential pharmacodynamic property of kratom[15,47]. At larger doses, kratom possesses unique narcotic qualities that blend psychostimulant and opiate-like effects[48]. Chronic usage of kratom has been linked to dependency[39].

PHARMACOLOGICAL EFFECTS OF KRATOM LEAVES

Consumption of 5-15 g of kratom leaves is believed to give opioid-like effects[24]. The euphoric effects begin around 10 min after consuming a few grams of dried leaves. At this dosage, kratom may give pain relief and alleviate symptoms of opioid withdrawal, with diarrhea as a possible side effect. Euphoria is more frequently attained at this higher level. Nevertheless, the effects are typically less powerful than with opioid medications[24]. Consumption of more than 15 g of kratom leaves could cause stupor, similar to the effects of opioids[24]. Most people will first suffer sweating, nausea, and dizziness. The early pleasure and tiredness are quickly replaced by a tranquil and dreamy state[44]. Tremors, anorexia, weight loss, convulsions, and psychosis have been reported in regular kratom users[6,24] who consumed high doses of kratom in a short period of time[6,24].

Synergistic effects of mitragynine and 7-hydroxymitragynine produce the analgesic effect desired by kratom users for self-treatment of pain and anxiety. Whilst these alkaloids exert sedative effects at high dosages (5-15 g), they exert stimulating effects at low levels (1-5 g)[44,47]. A dosage of 1-5 g of raw leaves is considered a low to moderate dose[16,24]. This dose is frequently associated with the stimulant effects frequently employed by laborers to combat weariness[24] and achieve greater work capacity while increasing attentiveness, sociability, and libido. Additionally, users may experience normal to slightly constricted pupils and blushing at this dosage. In general, adverse effects are mild. Nonetheless, anxiety and internal agitation have been reported[24]. Other effects of mitragynine included inhibition of ileum motility[7], smooth muscle contraction[49], and stomach acid production[50].

PHARMACOKINETICS AND DRUG-DRUG INTERACTIONS OF KRATOM

Kratom users should anticipate the full effects within 30-60 min after administration; however onset can occur as early as 10-20 min. Mitragynine and 7-hydroxymitragynine have half-lives of approximately 3.5 h and 2.5 h, respectively. Both are mostly removed from the body *via* urine[24,51]. The effects of kratom normally last between 5-7 h, with the biggest effects occurring between 2 and 4 h after administration. However, mild side effects can persist up to a day[24,43,52,53].

Kratom metabolism is primarily hepatic, and there is evidence that it can influence the metabolism and efficacy of other medicines by inducing drug-metabolizing enzymes, namely CYP450s and UDP-glucuronosyl transferase (UGT)[54]. The effects of kratom on human recombinant CYP450 enzyme activity have been studied in various research[55]. Herb-drug interactions were observed when mitragynine was used with herbal or modern medications that share the same metabolic pathway[56]. Mitragynine has a half-life of as little as 3 h, although it may be longer as suggested by others[57,58]. Significant advancement in kratom pharmacology conception revealed that mitragynine is transformed *in vivo via* hepatic metabolism into 7-hydroxymitragynine[59-61]. As a result, it has been hypothesized that 7-hydroxymitragynine is the active metabolite of mitragynine responsible for the majority, if not all, of the effects usually ascribed to the mitragynine precursor. Mitragynine is activated by CYP34A-mediated dehydrogenation, a mechanism akin to how opiates such as codeine are activated *via* CYP2D6-mediated dehydrogenation. In spite of the fact that 7-hydroxymitragynine is found in kratom extracts at minimal levels, the endogenous synthesis of 7-hydroxymitragynine from mitragynine was

significant[59,60,62].

In contrast to oral treatment, intravenous injection of mitragynine in rats was shown to be rapidly distributed to the peripheral compartments through systemic circulation or the central compartment[63, 64]. Mitragynine has a high intestinal permeability in rats. Mitragynine and 7-hydroxymitragynine can pass the blood-brain barrier and are dispersed throughout the brain. Mitragynine has a larger blood-brain barrier permeability and is more readily absorbed into brain tissue than 7-hydroxymitragynine[62, 65]. Mitragynine and 7-hydroxymitragynine inhibit P-glycoprotein[58,61]. These findings indicate that kratom not only penetrates the blood-brain barrier but also inhibits the brain from excreting other compounds *via* the P-glycoprotein efflux mechanism, hence enhancing the bioavailability of sensitive medicines.

Given the rise of reports on toxicity when used in combination with other drugs[66-70], it is worthwhile to investigate the pharmacological interactions of kratom. Drug-drug interactions by modulation of hepatic P450 activity and drug metabolism have been demonstrated in animal investigations[54,55]. Mitragynine appears to inhibit hepatic demethylases, transferases, and the glucuronidation reaction spurred by UGT like UGT2B7 and UGT1A1[71-74]. This has a major indication for the possibility of interaction of kratom and other UGT substrates, such as buprenorphine and ketamine, which are metabolized by UGT2B7[74]. These findings have been cited as a possible explanation for cases of toxicity associated with co-administration of kratom and other drugs, including a fatality associated with supratherapeutic doses of a prescription antipsychotic concomitant with kratom ingestion[67].

A proposed explanation for drug-drug interactions is the effect of kratom on the cytochrome P450 system, a set of enzymes involved in the metabolism of a wide variety of drugs[75]. Two of the most important enzymes involved in drug metabolism are CYP2D6 and CYP3A4. Mitragynine inhibits CYP2C9 and CYP2D6 in a noncompetitive manner and CYP3A4 competitively[56] indicating that kratom has tremendous interaction potential[75]. The largest inhibitory impact is observed for CYP2D6 and CYP3A4, indicating compounds that share the same metabolic route may contribute to unfavorable interactions[55,56]. Due to the inhibitory effects of kratom, substrates for these enzymes may accumulate, leading a typically safe dosage to reach hazardous levels. Thus, while one of kratom's active ingredients, 7-hydroxymitragynine, is mostly responsible for the herb's sedative and analgesic properties, the other active ingredient, mitragynine, may be the cause of unfavorable medication interactions *via* its influence on cytochrome P450 enzymes. It is obvious that identifying herbs as possible medication inhibitors may assist or limit the risk of adverse effects associated with herb–drug interactions[55].

BENEFIT AND RISK OF KRATOM USAGE

Concerns regarding the potential of kratom dependency and addiction in humans are well founded[30, 76,77]. However, for many frequent users, the primary objective was merely to avoid weariness and to boost energy. In such instances, frequent usage may not be defined as dependency or addiction but rather as a desire to increase productivity[78]. This is consistent with "drug instrumentation" hypotheses, according to which a substance is used for a specific, planned aim[6,79]. Long-term use of kratom may result in adaptation, where outright addiction was reported under certain circumstances [76]. It has been suggested that a considerable percentage of kratom usage happens as a substitution for more hazardous drugs, particularly opioids in individuals who already have a history of substance misuse. In these circumstances kratom use is considered harm reduction rather than drug abuse[6,80].

Apart from its misuse potential, kratom poses a slew of additional dangers to patients, mostly as a result of its status as an unregulated supplement. Nothing can be done to assure the veridicality, pureness, grade, and safety of commercially accessible kratom formulations in the absence of governmental control[81]. As a result, it is impossible to determine exactly what is contained in commercially available kratom formulations. Furthermore, the quantity of mitragynine can vary significantly[22]. There have been reports that kratom products can be enhanced in potency by intentionally raising the quantity of 7-hydroxymitragynine[82]. Additionally, many cases of purposeful adulteration of kratom have been observed, including the insertion of synthetic drugs such as phenylethylamine or O-desmethyltramadol, both resulting in patient fatalities[83,84]. Additional dangers include purposeful or accidental product contamination. Laboratory and epidemiological evidence in 2018 specified that kratom was the cause of salmonella infestation[85]. In addition, there have been instances of kratom products being sold that were later shown to have dangerous heavy metal impurities[12].

ADVERSE EFFECTS OF KRATOM USAGE

Kratom side effects, particularly for regular heavy kratom users, were agitation (18.6%), followed by tachycardia (16.9%), sleepiness (13.6%), and disorientation (8.1%)[86]. Seizures occurred in 6.1% of patients, hallucinations in 4.8%, and coma in 2.3%. Other symptoms include weight loss, frequent

urination, insomnia, fatigue, constipation, dry mouth, nausea, and hyperpigmentation of the cheeks[43,44]. Withdrawal symptoms due to the sole usage of kratom are too mild to be detected even for heavy users[44]. Apart from the initial adverse effects of kratom consumption, persistent and high-dose use results in various major side effects such as respiratory depression[66]. Injury to the liver, heart, lungs, kidneys, and neurological system are more significant and life-threatening adverse effects[87].

Concurrent use of kratom and other drugs has been associated with the development of focal and generalized tonic-clonic seizures, possibly as a result of the inhibitory effect of the active components of kratom on cytochrome P450 enzymes and P-glycoprotein[88]. Death was reported in 91 (59.9%) of 152 kratom-positive persons as documented by the unintentional drug overdose reporting system of the United States[89]. Co-administration of kratom and other medicines has the potential to enhance toxicity. A combination of mitragynine and morphine has been found to improve analgesia and delay the development of morphine tolerance in rats[90]. It has been reported that kratom extracts may alleviate symptoms of ethanol withdrawal by lowering alcohol consumption[91].

Muscle relaxation is a common physiological consequence of opiate usage and is frequently noted in kratom users[92]. Mitragynine and other kratom alkaloids may operate similarly to other opiates on the neuromuscular junction[92]. Mitragynine may also cause mild tremors and stiff fingers and toes[93]. This might be explained by the way stimulant and depressive effects are classified at low and high dosages, respectively. Seizures have been observed following kratom usage[92]. Intriguingly, seizures associated with kratom usage doubled in Thailand between 2005 and 2011[16].

A more recent study revealed that kratom caused hepatotoxicity in patients[94]. In addition, kratom-mediated liver damage, stomach pain, jaundice, pruritus, and dark urine were often reported as presenting signs and symptoms[95]. Autopsy results of kratom-related fatalities showed the presence of edema in the brain and lungs, as well as congestion in several organs[96].

A variety of organ systems can be affected due to kratom usage, which include kidney injury[97], cardiotoxicity and arrhythmia[98,99], thyroid injury and hypothyroidism[100], lung injury/acute respiratory distress syndrome[101,102], neonatal abstinence syndrome[103-107], and hepatic injury[108-111]. Amongst these, hepatic injury such as cholestatic hepatitis pattern similar to other drug-related injuries is frequently reported[112]. A number of neurological problems associated with kratom toxicity, including acute brain damage and coma, were documented[112].

Toxicity of kratom is dose-dependent, especially when kratom powder dosages surpass 8 g[86]. An overdose fatality from kratom alone is not common, although it has been reported in the United States and Southeast Asia[113]. This is in line with pharmacologic research and epidemiological investigations of kratom in Southeast Asia. Unlike morphine-like opioids, kratom does not cause life-threatening respiratory depression and is not linked to the personal and societal impairment that morphine-like opioids are linked to[3,8,113].

CONCLUSION

Kratom exerts its pharmacological effects in a dose-dependent manner, where it acts as a stimulant at low doses and a depressant at high doses. Regular usage of kratom can lead to dependency. The cellular mechanisms of kratom are complex and not well understood. The major alkaloid of the kratom leaves, mitragynine, and its minor alkaloid, 7-hydroxymitragynine, are likely responsible for the pharmacological effects of kratom. As the data have shown so far, deaths due to the sole use of kratom are rare. Typically, the combination use of kratom with other illicit drugs are the main causes of death. Given the valuable therapeutic properties of kratom, total banning of kratom will be a great loss to the pharmaceutical industry. Instead controlled usage should be practiced especially in the event of kratom misuse for recreational purposes. Considering both benefits and risks of kratom usage, one can wisely choose to use it for good.

FOOTNOTES

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ORIGINAL ARTICLE

Basic Study

Antidepressant-like potential of silymarin and silymarin-sertraline combination in mice: Highlighting effects on behaviour, oxidative stress, and neuroinflammation

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Abstract

BACKGROUND

Currently, there is increasing advocacy for the use of diet, dietary supplements, and herbal remedies in depression management.

AIM

To determine the antidepressant effects of standardized silymarin (SILY) extract either as a sole agent or as an adjunct in depression therapy.

METHODS

Adult mice were assigned into three main groups based on the neurobehavioural models; and each main group had ten treatment groups of 10 mice each. Treatment groups were: Vehicle control group, oral sertraline (SERT) group, two groups fed SILY)-supplemented diet (SILY at 140 and 280 mg/kg of feed, respectively), dexamethasone (DEX; *i.p.*) group, DEX/SERT group, two groups of DEX/SILY (SILY at 140 and 280 mg/kg of feed, respectively), and another two groups of (SERT/DEX/SILY) (SILY at 140 and 280 mg/kg of feed, respectively, plus *i.p.* DEX plus SERT). Duration of the study was 7 wk, and treatments were administered daily.

RESULTS

SILY (alone) increased body weight, open field locomotor activity, rearing, and grooming; it also enhanced spatial working memory while decreasing anxiety-related behaviours and behavioural despair. SILY also improved antioxidant status while decreasing lipid peroxidation, acetylcholinesterase activity, and

inflammatory markers. Neuronal integrity of the cerebral cortex and hippocampus was preserved. Overall, when administered alone or with SERT, SILY counteracted DEX-induced behavioural and biochemical changes while preserving neuromorphological integrity.

CONCLUSION

In conclusion, SILY is beneficial in mitigating DEX-induced central nervous system and other related changes in mice.

Key Words: Behavioural despair; Depression; Mental Health; Neurobehaviour; Neuromorphology

Core Tip: Depression is a neuropsychiatric disorder that has in recent times become a leading cause of disability and a major contributor to global disease burden and suicide. In recent times there has been increasing advocacy for the use of dietary supplements and herbal remedies in depression management. While antidepressant effects of extracts of silybum marianum seeds have been reported, there is a dearth of scientific information on the possible effect of its standardized silymarin extract either as a sole agent or as an adjunct in depression therapy.

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INTRODUCTION

Depression is a neuropsychiatric disorder that has in recent times become a leading cause of disability and a major contributor to global disease burden and suicide[1]. It is characterised by the presence of anhedonia and/or evidence of alterations in mood including irritability, sadness, or emptiness[2-6]. In the last decade or more, the global prevalence of depression has continued to rise[1,7], with depression accounting for approximately 12% of hospital admissions, 50% of mental health consultations, and 4% of suicides[6,8,9]. In addition to a high socioeconomic burden and significant morbidity/mortality, depression has been ranked as the single largest contributor to global disability and suicide deaths[3,5,10-13]. Scientific evidence[14,15] of the critical role of serotonin in the pathogenesis of depression was instrumental to the development of some of the current antidepressant drugs (fluoxetine and sertraline [SERT]) that selectively inhibit the reuptake of serotonin at serotonin transporters, and thereby increase serotonin concentration within the synaptic cleft[15,16]. While significant strides have been made in developing newer drugs for the management of depression, the obvious advantages of more tolerable, less toxic, and more affordable treatment options continue to spur researchers to do more.

In recent times, the impact of diet, dietary supplements, and herbal remedies in the maintenance of mental health, as well as the aetiology, progression, and management of mental illness is becoming important areas of research[17-19]. Specifically, the search for modifiable factors in depression has led to the study of the possible associations between the development of depressive illness and dietary patterns. A number of studies have been successful in demonstrating the value of diet and/or dietary supplements including selenium, zinc, and vitamins B, C, and K in the prevention, pathogenesis, or outcome of depression[20-25]. The antidepressant effects of extracts of parts of plants such as the *Silybum marianum* seed have also been reported[26].

Silymarin (SILY) is a polyphenolic antioxidant complex which is derived from the fruit and seeds of the 'milk-thistle' plant known as *Silybum marianum*. While this ancient medicinal plant has been used for centuries for hepatoprotection (or the management of hepatic disorders), the production of standardised fractions of the plant has allowed for a widespread research of its medicinal potential[27-29]. The antifibrotic, antioxidative, immunomodulatory, anti-inflammatory, and antinociceptive properties of SILY have been documented[30-33], and at pharmacological doses, it has been reported to be non-toxic[30,34]. A number of studies have also reported the neuroprotective effects of SILY in different animal models[26-28,35-37]. While there have been suggestions of the possible antidepressant effects of *Silybum marianum* extracts, there is a dearth of scientific information on the possible antidepressant effects of standardised formulations of SILY used alone or as an adjunct. Therefore, this study evaluated the effects of dietary supplementation with SILY, alone or in combination with SERT, on body weight, food intake, neurobehaviour, oxidative stress parameters, inflammatory markers, and acetylcholinesterase

levels in a dexamethasone (DEX) model of depression in mice.

MATERIALS AND METHODS

Drugs and chemicals

SILY (Silybon-70® Micronova Pharmaceutical Industries Ltd, Lagos Nigeria), SERT capsules (Zoloft® 50 mg, Pfizer Inc. Lagos, Nigeria), and DEX phosphate injection (4 mg/mL, Vixa Pharmaceutical Co. Ltd, Lagos, Nigeria) were obtained commercially. Assay kits for lipid peroxidation (malondialdehyde [MDA] assay kit), glutathione peroxidase (GPx), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (Biovision Inc. Milpitas, CA, United States) were obtained and refrigerated until used. All other chemicals were of analytical grade.

Animals

Adult male Swiss mice (Empire Breeders, Osogbo, Osun State, Nigeria) weighing between 18-25 g each were used for this study. Mice were housed singly in cages located in temperature-controlled quarters (22 °C-25 °C) with lights on at 7.00 a.m. daily. Animal diet was commercially sourced (TOP® feeds) standard rodent chow (29% protein, 13% fat, and 58% carbohydrate). Mice had access to food and water *ad libitum*, except during the behavioural tests. All procedures were conducted in accordance with the approved protocols of the Ladoke Akintola University of Technology and within the provisions for animal care and use prescribed in the scientific procedures on living animals European Council Directive (EU2010/63).

Feed

Animals were fed commercially available rodent diet [(standard diet (SD)] sourced from Top Feeds Ltd, Ibadan Nigeria). SILY was incorporated into standard rodent diet at 140 and 280 mg/kg of feed, respectively.

Experimental method

Adult male mice were randomly assigned into three main groups (1-3) based on the neurobehavioural models. Group 1 animals were exposed to the elevated plus maze and tail-suspension paradigm, group 2 were exposed to the Y-maze and forced-swim paradigm, while mice in group 3 were exposed to the open-field arena and radial arm maze. Animals in the main groups were subsequently assigned into ten treatment groups of 10 mice each. Treatment groups were: Vehicle control group [fed standard diet (SD) and given intraperitoneal (*i.p.*) saline plus oral saline], SERT group (fed SD and given *i.p.* saline plus oral SERT), two groups fed SILY-supplemented diet (at 140 and 280 mg/kg of feed, respectively; SILY 140 and SILY 280) and given *i.p.* saline plus oral saline, DEX group (fed SD and given *i.p.* DEX plus oral saline), DEX/SERT group (fed SD and given *i.p.* DEX plus oral SERT), two groups (DEX/SILY) fed SILY-supplemented diet (at 140 and 280 mg/kg of feed, respectively) and given *i.p.* DEX plus oral saline (DEX/SILY 140 and DEX/SILY 280), and another two groups fed SILY-supplemented diet (at 140 and 280 mg/kg of feed, respectively) and given *i.p.* DEX plus oral SERT (SERT/DEX/SILY 140 and SERT/DEX/SILY 2800). SERT was administered at 5 mg/kg[38], while DEX was administered at 4 mg/kg[39-41]. Total duration of the study was 7 wk, and all treatments were administered daily. Mice in all groups were weighed weekly (7.00 am, before feeding) and food intake was measured as previously described[42-44] using a weighing balance (Mettler Toledo Type BD6000, Greifensee, Switzerland). Food changes occurred daily at 8.00 am. Food hoppers that contained pre-weighed quantities of food were provided daily to the mice; a thin plastic sheet was placed beneath the cages to catch food spillage. Total food consumption was then measured as the difference between the pre-weighed standard chow and the weight of chow in hopper daily. Crumbs in the plastic sheets were weighed and accounted for in the measurement of total food consumed during the 24-h period[42]. At the end of the experimental period, animals were exposed to the respective paradigms. Twenty-four hours after the last behavioural test, animals in the open field and radial arm maze group were euthanised by cervical dislocation. Blood was taken for assessment of oxidative stress parameters and inflammatory markers (tumor necrosis factor (TNF)–α and interleukin-10). The hippocampus and cerebral cortex were excised and either homogenised for the assessment of inflammatory markers, antioxidant status, and acetylcholinesterase activity or processed for general histological examination.

Assessment of body weight and food intake

Body weight of animals in all groups were measured weekly using an electronic weighing balance (Mettler Toledo Type BD6000, Switzerland) while the amount of food consumed was measured daily. Relative change in body weight or food intake was calculated for each animal using the equation below following which results for all animals where computed to find the statistical mean.

Behavioural tests

Mice were transported in their home cages to the behavioural testing laboratory and allowed to acclimatise (10 min) before exposure to paradigms. Each animal was placed in the apparatus following which behaviours were recorded. On completion of the tests, each mouse was removed from the maze and returned to the respective home cages. The interior surfaces of the mazes were then cleaned with 70% ethanol and wiped dry to remove traces of conspecific odour. Behavioural parameters were then scored manually by independent observers who were blind to the groupings.

Anxiety model: Elevated plus maze

The elevated plus-maze (EPM) is a plus-shaped apparatus with four arms placed at right angles to each other. The EPM used in the study and the procedure are as previously described[42,45,46].

Open field

Ten minutes of locomotion, rearing, and grooming were observed in the open field and scored as previously described[47,48].

Tail suspension test

The tail suspension test (a measure of behavioural despair) was carried out according to the method described by Steru *et al*[49], Młyniec and Nowak[50], and Onaolapo *et al*[51]. Mice were securely fastened (using a medical adhesive tape) by the tip of their tail to a flat platform and suspended for 6 min approximately 30 cm below the platform. The total time of immobility was measured during the 6-min period of the testing session. Immobility, which was defined as the period the animal hung passively without limb movement, was scored[40].

Forced swim test

The forced swim test is a measure of behavioural despair in mice. The test was carried out according to the method described by Porsolt *et al*[52], Kroczka *et al*[53], and Onaolapo *et al*[51]. Mice were dropped individually into glass cylinders which had a height of 25 cm and diameter of 10 cm, were filled with 10 cm of water (water level was marked to ensure uniformity), and maintained at a temperature of 23-25 °C. The dimensions of the glass cylinder ensured that the mouse was unable to touch the bottom of the cylinders either with their feet or their tails, during the test. The height also prevented mice from escaping from the cylinder. Animals were then returned (they were dried with paper towels to prevent hypothermia) to their home cages after 15 min in water. They were reintroduced into the cylinders 24 h later. Mice were exposed to the forced swim paradigm for 6 min. The total duration of immobility was measured during the last 4 min of the forced swim test. The mouse was considered immobile when it had remained floating passively in the water.

Memory tests

The Y- and radial arm mazes were used to assess and score spatial working memory as previously described[54,55]. The Y-maze has three arms (41 cm long and 15 cm high, 5 cm wide at an angle of 120°), while the radial arm maze apparatus has 8 arms measuring 33 cm long spaced equidistantly from each other.

Blood collection

Blood collected from each mouse *via* cardiac puncture was used for the estimation of lipid peroxidation, GSH, SOD, and GPx. Samples were collected into unheparinised bottles and processed as previously described[56,57].

Brain homogenization

Within 24 h of the completion of the behavioural tests, animals in all groups were euthanised by cervical dislocation post-anaesthesia with diethyl ether. Homogenates of the hippocampus and cerebral cortex were prepared in ice-cold phosphate buffered saline, using a Teflon-glass homogeniser. The homogenate was centrifuged at 5000 rpm at 4 °C for 15 mi. The supernatant obtained was then used for estimation of lipid peroxidation levels and antioxidant status.

Biochemical assays

Estimation of MDA content (lipid peroxidation): Lipid peroxidation level was measured as MDA content as previously described[58]. Change in colour was measured at 532 nm. The MDA kit used had a detection range of 7.813-500 ng/mL and a sensitivity < 4.688 ng/mL. The intra-assay coefficient of variability was < 7%, and the inter-assay coefficient of variability was < 9%.

Antioxidant activity

SOD activity was determined using a commercially available assay kit. Colour changes were measured at an absorbance of 560 nm as described previously[29,58]. The activity of SOD is expressed in

units/mL.

Levels of GSH were determined following the instructions of the manufacturer. A yellow-coloured complex which can be measured at an absorbance of 412 nm is formed by GSH form when it reacts with Ellmans reagent (DTNB). Levels of GSH are expressed in nmol/mL.

GPx is an enzyme that catalyses the reduction of hydroperoxides, such as hydrogen peroxide. GPx activity was determined as previously described[29]. The activity of GPx is expressed in units/mL.

Tumour necrosis factor-α and interleukin-10

Tumour necrosis factor-α and interleukin (IL)-10 were measured using enzyme-linked immunosorbent assay (ELISA) techniques with commercially available kits (Enzo Life Sciences Inc. NY, United States) designed to measure the 'total' (bound and unbound) amount of the respective cytokines.

Acetylcholinesterase activity

Brain acetylcholinesterase activity (Biovision, United States) was determined using commercially available assay kits following the instructions of the manufacturer.

Tissue histology

Sections of the cerebral cortex and hippocampus were fixed in 10% formal saline for 24 h, processed for paraffin wax embedding, dehydration, clearing, and infiltration, sectioned, and then mounted following which they were processed for general histological staining using haematoxylin and eosin as previously described[40].

Statistical analysis

Data were analysed using Chris Rorden's analysis of variance (ANOVA) for windows, version 0.98. Data analyses were done by ANOVA, and post-hoc test (Tukey HSD) was used for within and between group comparisons. Results are expressed as the mean ± SEM. $P < 0.05$ was taken as the accepted level of significant difference from control or standards.

RESULTS

Effect of silymarin on body weight

Figure 1 shows the effect of SILY on the change in body weight. There was a significant [F (9, 90) = 48.1, $P < 0.001$] decrease in body weight in the groups administered with SERT, DEX, DEX/SERT, and DEX/SILY 140, while an increase in body weight was observed in groups administered with SILY 140 and SILY 280, DEX/SILY 280, and those administered with *i.p*. DEX, oral SERT, DEX/SERT/SILY 140, and DEX/SERT/SILY 280 compared to the vehicle control. Compared to SERT alone, there was a significant increase in body weight with SILY 280. While compared to DEX, body weight increased significantly with DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with the group administered with DEX/SERT, body weight increased significantly with DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY administered alone increased body weight compared to the vehicle control or SERT. SILY when administered alone (at 280 mg/kg) reversed DEX-induced changes in body weight. When co-administered with SERT, SILY at both concentrations reversed the changes in body weight induced by DEX.

Effect of silymarin on food intake

Figure 2 shows the effect of SILY on the change in food intake. There was a significant [F (9, 90) = 513, $P < 0.001$] decrease in food intake with DEX, DEX/SERT, DEX/SILY 140, and DEX/SILY 280, while an increase in food intake was observed with DEX/SERT/SILY 140 and DEX/SERT/SILY 280, compared to the vehicle control. Compared to SERT alone, there was no significant difference in food intake in any of the SILY alone groups. While compared to DEX, food intake increased significantly with DEX/SERT/SILY 140 and DEX/SERT/SILY 280. Compared with the group administered with DEX/SERT, food intake increased significantly with DEX/SERT/SILY 140 and DEX/SERT/SILY 280. Overall, the results showed that SILY administered alone did not significantly alter food intake compared to the vehicle control, SERT, or DEX, although co-administration of SILY with SERT was associated with an increase in food intake compared to the vehicle control, DEX, or DEX with SERT.

Effect of silymarin on locomotor and rearing activity

Figure 3 shows the effect of SILY on locomotor activity (upper panel) and rearing (lower panel). There was a significant [F (9, 90) = 26.5, $P < 0.001$] increase in locomotor activity with SILY 140, DEX/SILY 140, and DEX/SILY 280, and a decrease in locomotor activity with DEX compared to the vehicle control. Compared to SERT alone, there was a significant increase in locomotor activity with SILY 140. While compared to DEX, locomotor activity increased significantly with DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with the group

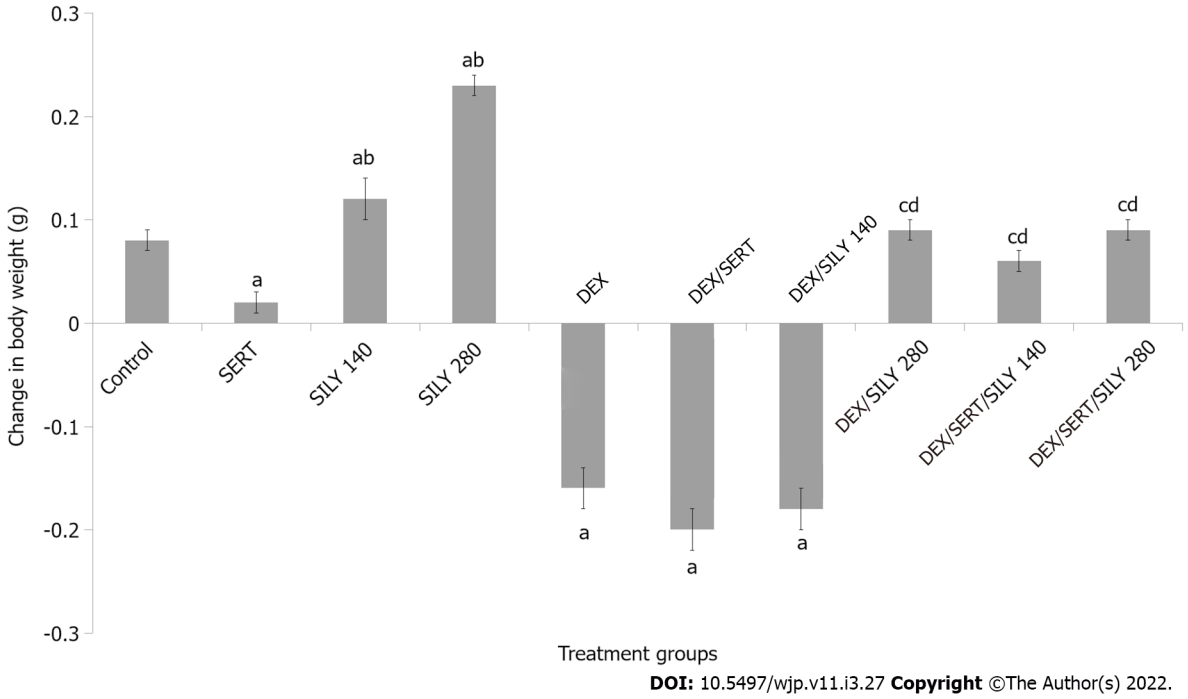

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Figure 1 Effect of silymarin on change in body weight. Each bar represents the mean ± SEM, [a]*P* < 0.05 *vs* control, [b]*P* < 0.05 vs SERT, [c]*P* < 0.05 *vs* DEX, [d]*P* < 0.05 *vs* DEX/SERT. SERT: Sertraline; DEX: Dexamethasone; SILY: Silymarin. Number of mice per treatment group = 10.

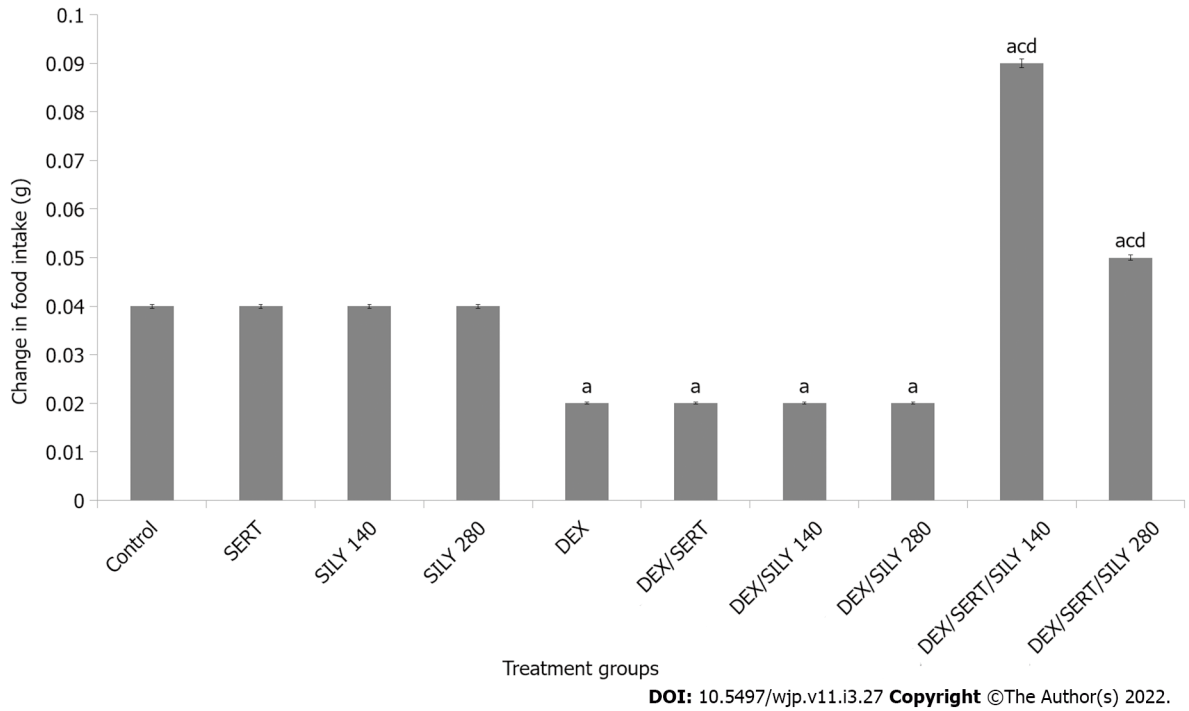

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Figure 2 Effect of silymarin on changes in food intake. Each bar represents the mean ± SEM, [a]*P* < 0.05 *vs* control, [b]*P* < 0.05 *vs* SERT, [c]*P* < 0.05 *vs* DEX, [d]*P* < 0.05 *vs* DEX/SERT. SERT: Sertraline; DEX: Dexamethasone; SILY: Silymarin. Number of mice per treatment group = 10.

administered with DEX/SERT, locomotor activity increased significantly with DEX/SILY 280 mg. Overall, the results showed that SILY (administered alone) concentration-dependently increased locomotor activity compared to the vehicle control and SERT. SILY alone or co-administered with SERT also mitigated the decrease in locomotor activity induced by DEX.

Rearing activity decreased significantly [F (9, 90) = 6.20, P < 0.001] with DEX and increased with DEX/SILY 140 and DEX/SILY 280, compared to the vehicle control. Compared to SERT alone, there was a significant increase in rearing activity with SILY 140. While compared to DEX, rearing activity

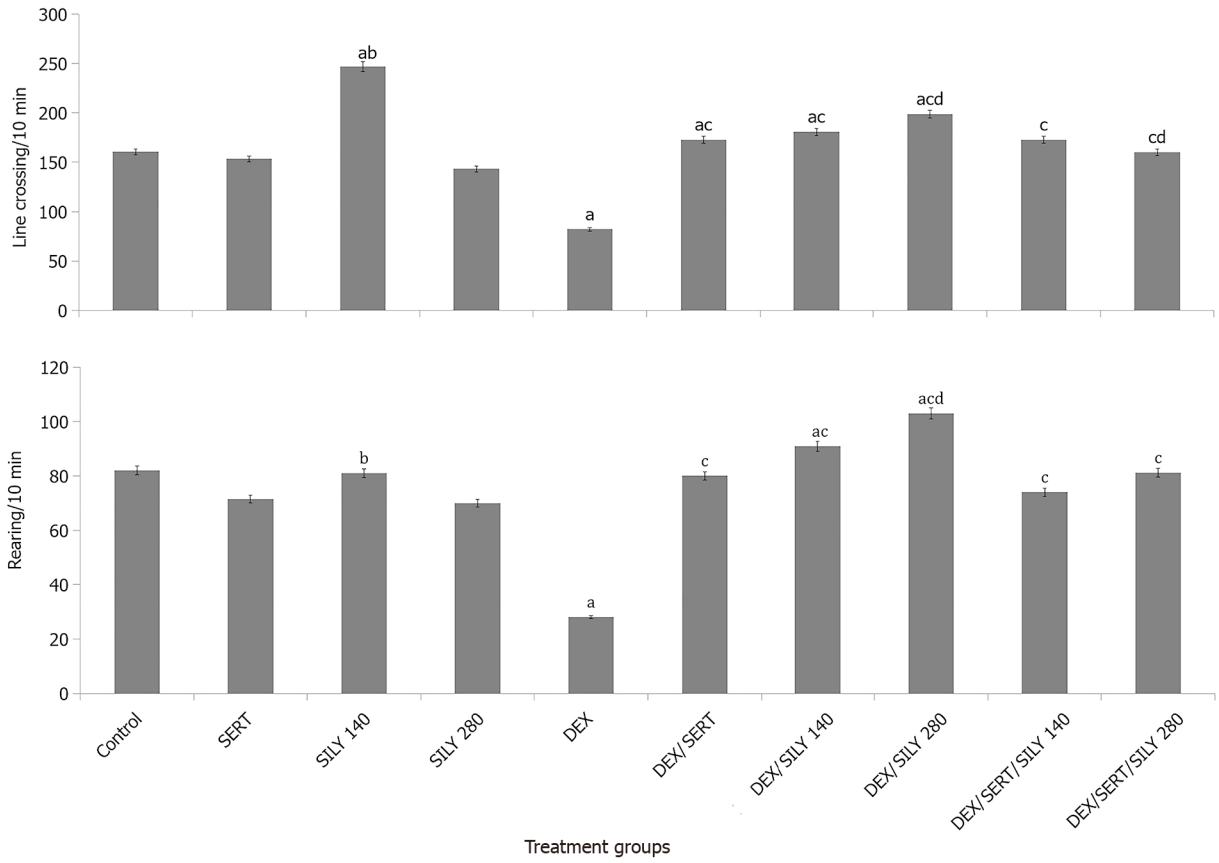

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Figure 3 Effect of silymarin on locomotor activity (upper panel) and rearing activity (lower panel). Each bar represents the mean ± SEM, [a]*P* < 0.05 *vs* control, [b]*P* < 0.05 *vs* SERT, [c]*P* < 0.05 *vs* DEX, [d]*P* < 0.05 *vs* DEX/SERT. SERT: Sertraline; DEX: Dexamethasone; SILY: Silymarin. Number of mice per treatment group = 10.

increased significantly with DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared to DEX/SERT, the rearing activity increased significantly with DEX/SILY 280. Overall, the results showed that SILY alone or co-administered with SERT also mitigated the decrease in rearing activity induced by DEX.

Effect of silymarin on grooming behaviour

Figure 4 shows the effect of SILY on self-grooming behaviour. There was a significant [F (9, 90) = 5.24, P < 0.001] increase in self-grooming with SILY, DEX/SILY, and DEX/SERT/SILY 140, while a decrease in self-grooming was observed with DEX and DEX/SERT compared to the vehicle control. Compared to SERT alone, there was a significant increase in self-grooming with SILY 140. While compared to DEX, self-grooming behaviour increased significantly with DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with the group administered with DEX/SERT, self-grooming increased significantly with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY administered alone concentration-dependently increased self-grooming behaviour compared to the vehicle control and SERT. SILY alone or co-administered with SERT also mitigated the decrease in self-grooming behaviour induced by DEX.

Effect of silymarin on spatial working memory in the Y- and radial arm mazes

Figure 5 shows the effect of SILY on radial arm (upper panel) and Y- (lower panel) maze spatial working memory tasks. There was a significant [F (9, 90) = 9.20, P < 0.001] increase in working memory with SILY 140, SILY 280, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, while a decrease in memory was observed with DEX compared to the vehicle control. Compared to SERT alone, there was a significant increase in working memory with SILY 140 and SILY 280. While compared to DEX, working memory increased significantly with DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with the group administered with DEX/SERT, working memory increased significantly with DEX/SILY 140, DEX/SILY 280, DEX/ SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY administered alone increased spatial working memory scores in the radial arm maze, compared to the

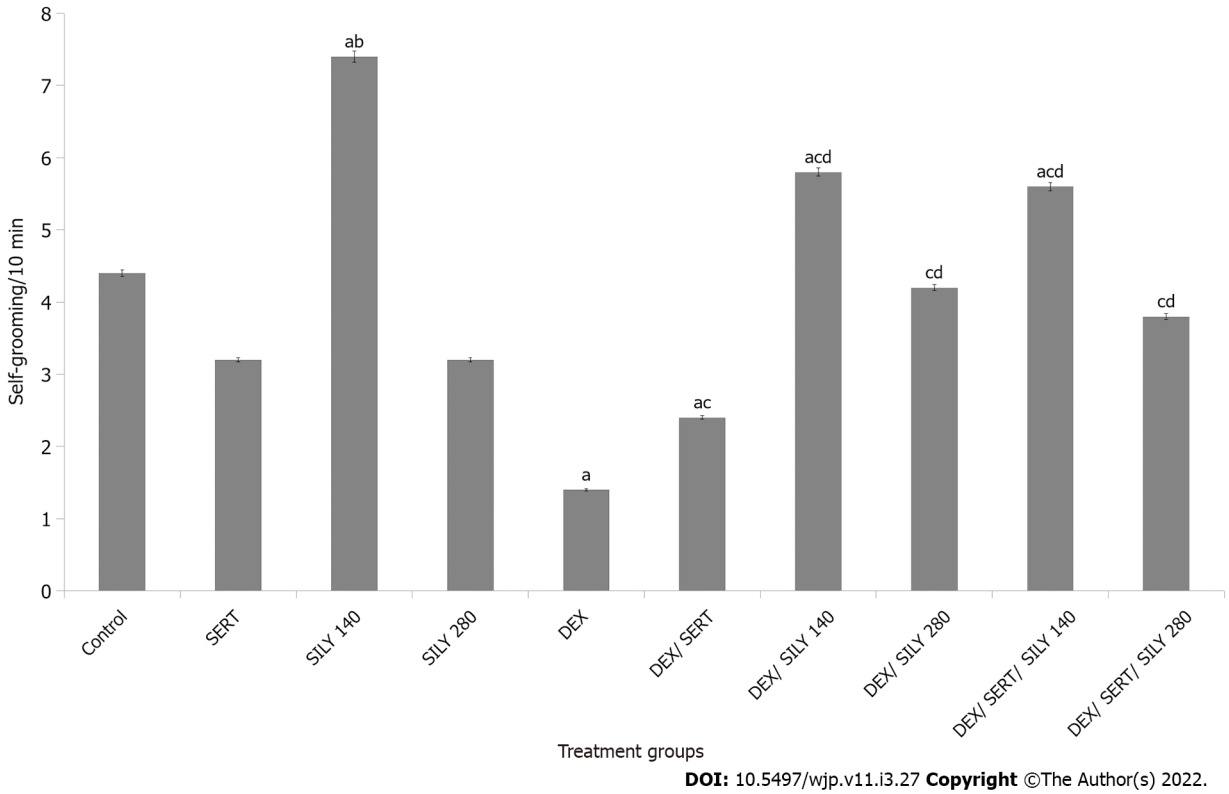

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Figure 4 Effect of silymarin on self-grooming. Each bar represents the mean ± SEM, $^{c}P < 0.05$ *vs* control, $^{b}P < 0.05$ *vs* SERT, $^{c}P < 0.05$ *vs* DEX, $^{d}P < 0.05$ *vs* DEX/SERT. SERT: Sertraline; DEX: Dexamethasone; SILY: Silymarin. Number of mice per treatment group = 10.

vehicle control and SERT. SILY alone or co-administered with SERT also counteracted the decrease in spatial working memory score induced by DEX.

Y maze spatial working memory increased significantly [F (9, 90) = 16.04, $P < 0.001$] with SILY 140, SILY 280, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, and decreased with DEX compared to the vehicle control. Compared to SERT alone, there was no significant difference in working memory in any of the groups fed SILY alone. While compared to DEX, working memory increased significantly with DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared to DEX/SERT, working memory increased significantly with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY administered alone improved spatial working memory scores in the Y-maze compared to the vehicle control. SILY alone or co-administered with SERT also counteracted the decrease in spatial working memory induced by DEX.

Effect of silymarin on anxiety-related behaviours

Figure 6 shows the effect of SILY on the time spent in the open (upper panel) and closed (lower panel) arms of the elevated plus maze. There was a significant [F (9, 90) = 15.11, $P < 0.001$] increase in open arm time with SERT, SILY 140, SILY 280, DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, while a decrease was observed with DEX compared to the vehicle control. Compared to SERT alone, there was a significant increase in open arm time with SILY 280. While compared to DEX, open arm time increased significantly with DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with the group administered with DEX/SERT, open arm time increased significantly with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY administered alone increased the time spent in the open arm of the EPM compared to the vehicle control. SILY alone or co-administered with SERT also mitigated the decrease in open arm time induced by DEX.

Time spent in the closed decreased significantly [F (9, 90) = 8.21, $P < 0.001$] with SERT, SILY 140, SILY 280, DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, and increased with DEX compared to the vehicle control. Compared to SERT alone, there was no significant difference in closed arm time in any of the groups fed SILY alone. While compared to DEX, closed arm time decreased significantly with DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with DEX/SERT, the time spent in the closed arm decreased significantly with DEX/SERT/SILY 280. Overall, the results showed that SILY administered alone decreased time spent in the closed arm compared to the vehicle control. SILY alone or co-administered

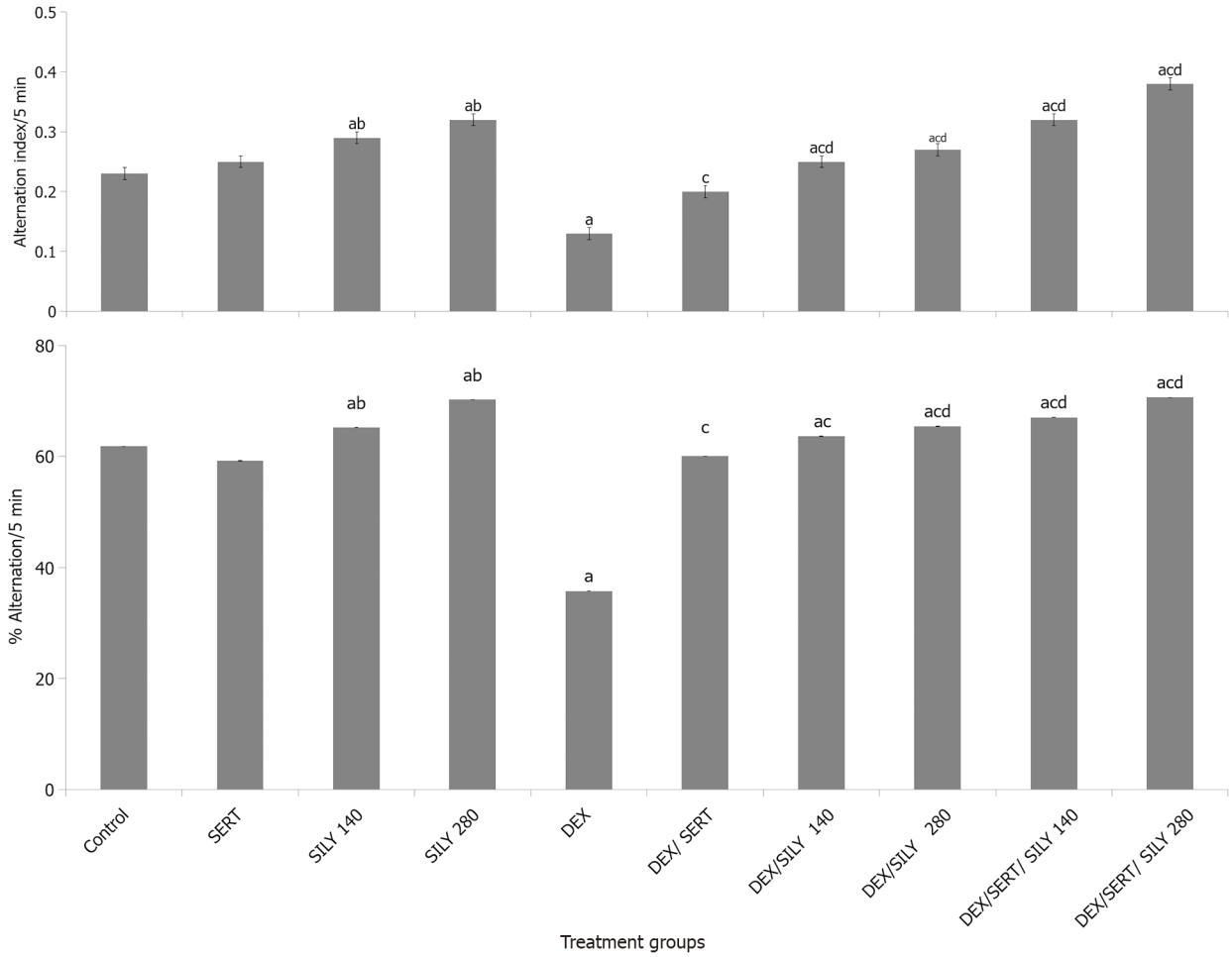

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Figure 5 Effect of silymarin on radial arm maze (upper panel) and Y–maze (lower panel) spatial working memory. Each bar represents the mean ± SEM, [a]*P* < 0.05 *vs* control, [b]*P* < 0.05 *vs* SERT, [c]*P* < 0.05 *vs* DEX, [d]*P* < 0.05 *vs* DEX/SERT. SERT: Sertraline; DEX: Dexamethasone; SILY: Silymarin. Number of mice per treatment group = 10.

with SERT also decreased time spent in the closed arm compared to DEX.

Effect of silymarin on behavioural despair

Figure 7 shows the effect of SILY on immobility time in the tail suspension (upper panel) and forced swim (lower panel) tests. There was a significant [F (9, 90) = 26.9, P < 0.001] decrease in immobility time with SILY 140, SILY 280, DEX/SERT, and DEX/SERT/SILY 140, and DEX/SERT/SILY 280 while an increase was observed with SERT, DEX, DEX/SILY 140, and DEX/SILY 280 compared to the vehicle control. Compared to SERT alone, there was a significant decrease in immobility time with SILY 140 and SILY 280. While compared to DEX, the immobility time decreased significantly with DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with the group administered with DEX/SERT, the immobility time decreased significantly with EX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY administered alone decreased immobility time compared to the vehicle control and SERT. SILY alone or co-administered with SERT also mitigated the increase in immobility time induced by DEX.

Immobility time in the forced swim test decreased significantly [F (9, 90) = 24.0, p < 0.001] with SERT, SILY 140, SILY 280, DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, and increased with DEX, compared to the vehicle control. Compared to SERT alone, there was a significant decrease in immobility time with SILY 140. While compared to DEX, the immobility time decreased significantly with DEX/SERT, DEX/SILY 140, and DEX/SERT/SILY. Compared to DEX/SERT, the immobility time decreased significantly with DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY administered alone decreased immobility time compared to the vehicle control and SERT. SILY alone or co-administered with SERT also mitigated the increase in immobility time induced by DEX.

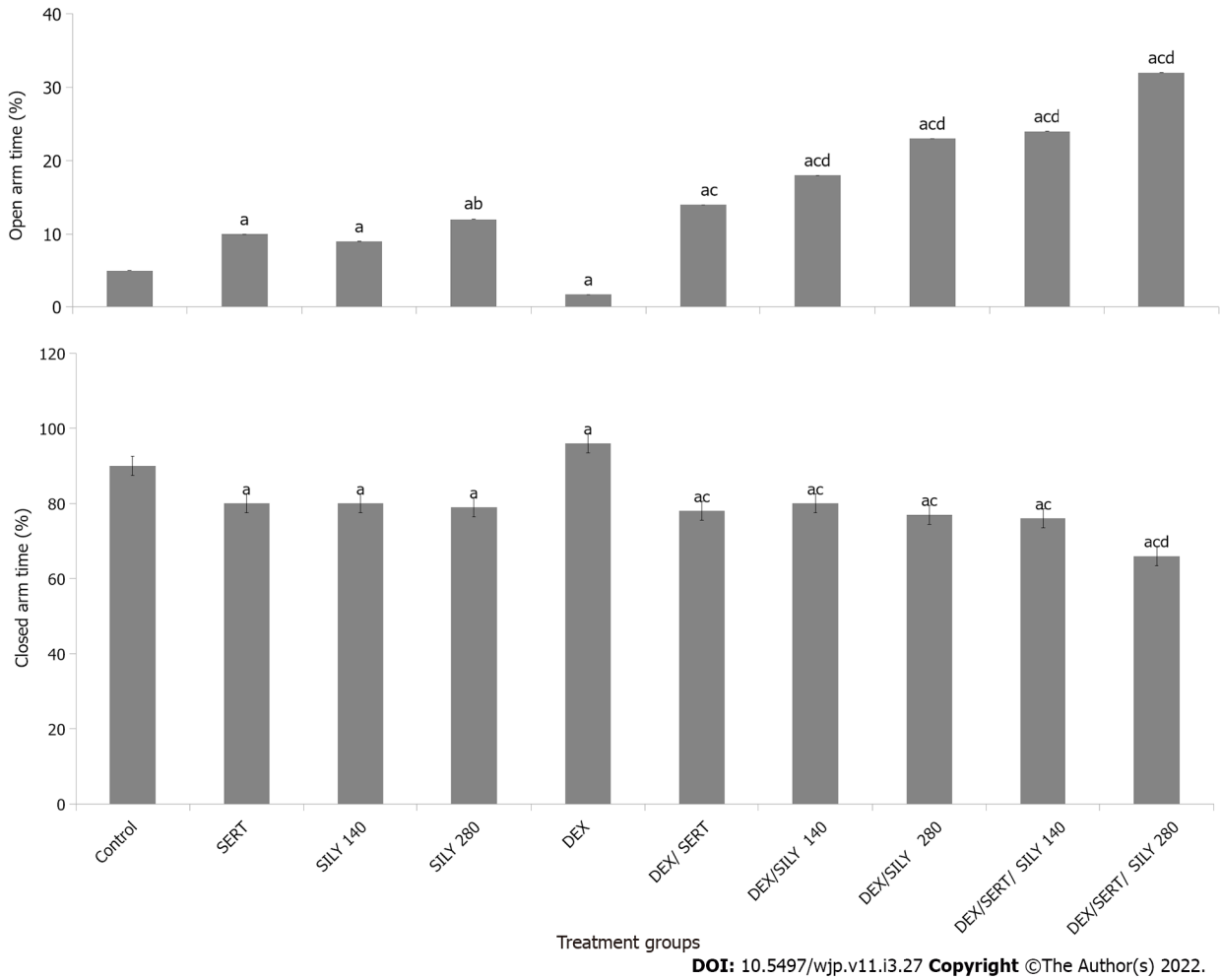

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Figure 6 Effect of silymarin on time spent in the open-arm (upper panel) and closed arm (lower panel) of the elevated plus maze. Each bar represents the mean ± SEM, [a]*P* < 0.05 *vs* control, [b]*P* < 0.05 *vs* SERT, [c]*P* < 0.05 *vs* DEX, [d]*P* < 0.05 *vs* DEX/SERT. SERT: Sertraline; DEX: Dexamethasone; SILY: Silymarin. Number of mice per treatment group = 10.

Effect of silymarin on serum lipid peroxidation and antioxidant status

Table 1 shows the effect of SILY on serum lipid peroxidation and antioxidant status. SOD [F (9, 90) = 13.11, P < 0.001], increased significantly with SILY 140, SILY 280, and DEX/SILY 280, while a decrease was observed with DEX, DEX/SERT, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, compared to the vehicle control. Compared to SERT alone, there was a significant increase with SILY 140 and SILY 280. While compared to DEX, there was an increase with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, and decrease with DEX/SERT. Compared to DEX/SERT, there was an increase in SOD activity with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Catalase [F (9, 90) = 25.32, P < 0.001] increased significantly with SILY 140, SILY 280, and DEX/SILY 280, while a decrease was observed with DEX, DEX/SERT, and DEX/SERT/SILY 140 compared to the vehicle control. Compared to SERT alone, there was a significant increase with SILY 140 and SILY 280. While compared to DEX, there was an increase with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared to DEX/SERT, there was an increase in catalase activity with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280.

GSH [F (9, 90) = 9.23, P < 0.001] increased significantly with SILY 140 and SILY 280, DEX/SILY 280, and DEX/SERT/SILY 280, while a decrease was observed with DEX and DEX/SERT 140 compared to the vehicle control. Compared to SERT alone, there was a significant increase with SILY 140 and SILY 280. While compared to DEX, there was an increase with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared to DEX/SERT, there was an increase in GSH levels with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280.

GPx activity [F (9, 90) = 10.32, P < 0.001] increased significantly with SILY 140 and SILY 280 and decreased with DEX and DEX/SERT compared to the vehicle control. Compared to SERT alone, there was a significant increase with SILY 140 and SILY 280. While compared to DEX, there was an increase with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared to DEX/SERT, there was an increase in GPx levels with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY

Table 1 Serum antioxidant status and lipid peroxidation level

Group	SOD (U/mL)	CAT (U/mL)	GSH nmol/mL	GPx IU/L	MDA μmol/L
Control	0.92 ± 0.02	23.12 ± 0.21	0.73 ± 0.10	11.68 ± 1.10	6.42 ± 0.02
SERT	0.93 ± 0.02	21.11 ± 0.20	0.70 ± 0.11	10.60 ± 2.01	5.40 ± 0.02
SILY 140	1.54 ± 0.11[a,b]	28.72 ± 0.23[a,b]	0.82 ± 0.05[a,b]	24.54 ± 0.34[a,b]	3.38 ± 0.04[a]
SILY 280	1.98 ± 0.02[a,b]	31.76 ± 0.22[a,b]	1.12 ± 0.05[a,b]	30.20 ± 0.78[a,b]	2.33 ± 0.03[a,b]
DEX	0.78 ± 0.03[a]	12.78 ± 0.22[a]	0.30 ± 0.05[a]	6.65 ± 1.10[a]	14.40 ± 0.06[a]
DEX/SERT	0.67 ± 0.01[a,c]	13.67 ± 0.20[a]	0.35 ± 0.01[a,c]	6.92 ± 1.00[c]	14.57 ± 0.16[a]
DEX/SILY 140	0.98 ± 0.01[c,d]	22.16 ± 0.10[c,d]	0.56 ± 0.04[a,c]	11.40 ± 1.23[c,d]	9.65 ± 0.05[a,c,d]
DEX/SILY 280	1.00 ± 0.12[a,c,d]	27.20 ± 0.22[a,c,d]	0.78 ± 0.03[a,c,d]	12.45 ± 1.30[c,d]	5.57 ± 0.01[a,c,d]
DEX/SERT/SILY 140	0.82 ± 0.02[a,c,d]	19.70 ± 0.10[a,c,d]	0.75 ± 0.01[c,d]	10.12 ± 1.01[c,d]	4.98 ± 0.05[a,c,d]
DEX/SERT/SILY 280	0.98 ± 0.02[a,c,d]	21.21 ± 0.20[c,d]	0.88 ± 0.03[a,c,d]	11.32 ± 0.76[c,d]	4.82 ± 0.02[a,c,d]

Values are expressed as the mean ± SEM.
[a]$P < 0.05$ *vs* control.
[b]$P < 0.05$ *vs* SERT.
[c]$P < 0.05$ *vs* DEX.
[d]$P < 0.05$ *vs* DEX/SERT. SOD: Superoxide dismutase; GPx: Glutathione peroxidase; MDA: Malondialdehyde; SERT: Sertraline; DEX: Dexamethasone; SILY: Silymarin. Number of mice per treatment.

140, and DEX/SERT/SILY 280.

Overall, the results showed that SILY administered alone or co-administered with SERT had a mixed response with regards to antioxidant status.

Lipid peroxidation measured as MDA levels decreased significantly [F (9, 90) = 6.19, $P < 0.001$] with SILY 140, SILY 280, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, while an increase was observed with DEX, DEX/SERT, and DEX/SILY 140 compared to the vehicle control. Compared to SERT alone, there was a significant decrease in MDA levels with SILY 140 and SILY 280. While compared to DEX, there was a decrease with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with the group administered with DEX/SERT, there was a decrease in MDA levels with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY administered alone or co-administered with SERT decreased lipid peroxidation levels.

Effect of silymarin on brain levels of inflammatory markers, acetylcholinesterase activity, lipid peroxidation, and antioxidant status

Table 2 shows the effect of SILY on brain (hippocampus and cerebral cortex) levels of inflammatory markers (TNF-α and IL-10), acetylcholinesterase activity, lipid peroxidation, and antioxidant status. Brain (hippocampus and cerebral cortex) levels of TNF-α [F (9, 90) = 65.12, $P < 0.001$] decreased significantly with SERT, SILY 140, SILY 280, DEX, DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, compared to the vehicle control. Compared to SERT alone, there was a significant increase with SILY 140 and SILY 280. While compared to DEX, there was an increase in brain (hippocampus and cerebral cortex) levels of TNF-α with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with the group administered with DEX/SERT, there was an increase in brain levels of TNF-α with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY administered alone decreased TNF-α levels, and when given alone or co-administered with SERT, it mitigated DEX-induced alterations in TNF-α levels.

Brain (hippocampus and cerebral cortex) levels of IL-10 [F (9, 90) = 22.36, $P < 0.001$] decreased significantly with SERT, DEX, DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, compared to the vehicle control. Compared to SERT alone, there was a significant increase with SILY 140 and SILY 280. While compared to DEX, there was an increase in brain levels of IL-10 with DEX/SILY and DEX/SERT/SILY at 140 and 280 mg/kg of feed, respectively. Compared with the group administered with DEX/SERT, there was an increase in brain levels of IL-10 with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY increased IL-10 Levels; alone or co-administered with SERT, it mitigated DEX-induced alteration in IL-10 Levels.

Table 2 Brain levels of inflammatory markers, acetylcholinesterase activity, lipid peroxidation, and antioxidant status

Group	TNF-α ng/g/protein	IL-10 pg/mg/protein	ACHe nmol/mg	MDA nmol/g/protein	GSH nmol/mg/protein	GPx mU/mg/protein
Control	38.78 ± 0.20	23.89 ± 0.20	32.10 ± 1.30	7.95 ± 0.50	0.75 ± 0.10	18.68 ± 1.10
SERT	24.12 ± 0.10[a]	19.20 ± 0.16[a]	28.19 ± 1.03[a]	8.01 ± 0.51	0.73 ± 0.11	17.60 ± 1.01
SILY 140	34.18 ± 0.10[a]	23.65 ± 0.20	24.22 ± 1.15[a,b]	6.91 ± 0.70[a]	0.92 ± 0.05[a,b]	34.54 ± 0.44[a,b]
SILY 280	33.11 ± 0.20[a]	23.80 ± 0.30	20.18 ± 1.15[a,b]	5.83 ± 0.63[a,b]	1.23 ± 0.05[a,b]	46.20 ± 0.54[a,b]
DEX	18.78 ± 0.13[a]	9.07 ± 0.10[a]	52.10 ± 1.25[a]	18.20 ± 0.56[a]	0.30 ± 0.05[a]	10.15 ± 0.80[a]
DEX/SERT	19.40 ± 0.10[a,c]	8.21 ± 0.19[a]	42.30 ± 1.11[a,c]	18.25 ± 0.76[a,c]	0.32 ± 0.02[a]	9.89 ± 0.80[c]
DEX/SILY 140	25.22 ± 0.11[c,d]	15.22 ± 0.20[c,d]	33.22 ± 1.24[c,d]	7.60 ± 0.80[c,d]	0.64 ± 0.03[a,c]	12.40 ± 0.83[a,c,d]
DEX/SILY 280	29.00 ± 0.12[a,c,d]	19.21 ± 0.23[c,d]	30.17 ± 1.13[a,c,d]	6.57 ± 0.63[a,c,d]	0.88 ± 0.03[c,d]	14.35 ± 0.07[a,c,d]
DEX/SERT/SILY 140	23.23 ± 0.10[a,c,d]	14.10 ± 0.12[a,c,d]	28.12 ± 1.21[c,d]	6.38 ± 0.61[a,c,d]	0.78 ± 0.01[a,c,d]	13.42 ± 0.71[a,c,d]
DEX/SERT/SILY 280	25.12 ± 0.10[a,c,d]	16.19 ± 0.15[a,c,d]	24.20 ± 1.10[a,c,d]	6.32 ± 0.50[a,c,d]	0.88 ± 0.03[a,c,d]	15.42 ± 0.89[a,c,d]

Values are expressed as the mean ± SEM.

[a]$P < 0.05$ *vs* control.

[b]$P < 0.05$ *vs* SERT.

[c]$P < 0.05$ *vs* DEX.

[d]$P < 0.05$ *vs* DEX/SERT. GPx: Glutathione peroxidase; MDA: Malondialdehyde; SERT: Sertraline; DEX: Dexamethasone; SILY: Silymarin. Number of mice per treatment.

Brain (hippocampus and cerebral cortex) acetylcholinesterase activity decreased significantly [F (9, 90) = 10.21, $P < 0.001$] with SERT, SILY 140, SILY 280, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, and increased acetylcholinesterase activity with DEX and DEX/SERT compared to the vehicle control. Compared to SERT alone, there was a significant decrease in brain acetylcholinesterase activity with SILY 140 and SILY 280. While compared to DEX, a significant decrease in brain acetylcholinesterase activity was observed with DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with the group administered with DEX/SERT, there was a decrease in brain acetylcholinesterase activity with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY decreased acetylcholinesterase activity; alone or co-administered with SERT, it mitigated DEX-induced alteration in acetylcholinesterase activity.

Brain (hippocampus and cerebral cortex) MDA levels decreased significantly [F (9, 90) = 10.21, $P < 0.001$] with SILY 140, SILY 280, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, and increased with DEX and DEX/SERT compared to the vehicle control. Compared to SERT alone, there was a significant decrease with SILY 140 and SILY 280. While compared to DEX, there was a decrease in brain MDA levels with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with the group administered with DEX/SERT, there was a decrease in MDA levels with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY decreased MDA levels; alone or co-administered with SERT, it mitigated DEX-induced alteration in MDA levels.

Brain (hippocampus and cerebral cortex) levels of GSH [F (9, 90) = 5.12, $P < 0.001$] increased significantly with SILY 140, SILY 280, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280 and decreased with DEX, DEX/SERT, and DEX/SILY 140 compared to the vehicle control. Compared to SERT alone, there was a significant increase with SILY 140 and SILY 280. While compared to DEX, there was an increase with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with the group administered with DEX/SERT, there was an increase in GSH with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280.

GPx activity [F (9, 90) = 6.27, $P < 0.001$] increased significantly with SILY 140 and SILY 280 and decreased with DEX, DEX/SERT, DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280, compared to the vehicle control. Compared to SERT alone, there was a significant increase with SILY 140 and SILY 280. While compared to DEX, there was a decrease with DEX/SERT and an increase with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Compared with the group administered with DEX/SERT, there was an increase in GPx with DEX/SILY 140, DEX/SILY 280, DEX/SERT/SILY 140, and DEX/SERT/SILY 280. Overall, the results showed that SILY increased GPx and GSH activity; alone or co-administered with SERT, it mitigated DEX-induced alterations in GPx and GSH activity.

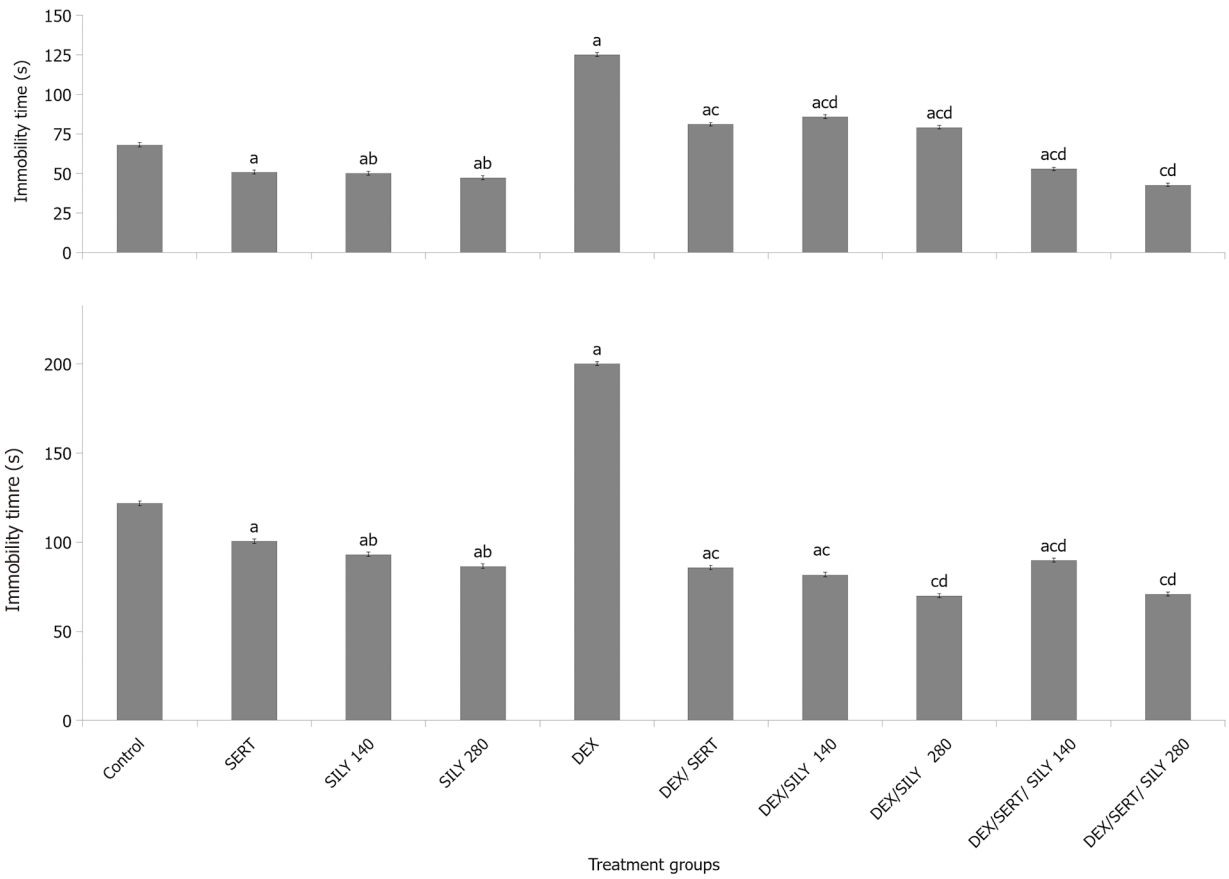

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Figure 7 Effect of silymarin on immobility time in the tail suspension (upper panel) and forced swim (lower panel) tests. Each bar represents the mean ± SEM, [a]*P* < 0.05 *vs* control, [b]*P* < 0.05 *vs* SERT, [c]*P* < 0.05 *vs* DEX, [d]*P* < 0.05 *vs* DEX/SERT. SERT: Sertraline; DEX: Dexamethasone; SILY: Silymarin. Number of mice per treatment group = 10.

Effect of silymarin on cerebral cortex and hippocampal morphology

Figure 8 shows representative photomicrographs of haematoxylin and eosin stained sections of the mouse cerebral cortex. Examination of the cerebral cortex sections of mice in the vehicle control group revealed characteristic architecture of the mouse cerebral cortex showing multipolar shaped pyramidal cells with rounded vesicular nuclei, granule cells visible as circular shaped neurons with large open-face nuclei, prominent nucleoli, and scanty cytoplasm and small round-vesicular shaped glial neurons interspersed within a pink-staining neuropil. These features are in keeping with normal cerebral cortex histology. Examination of the cerebral cortex sections of the SERT, SILY 140, and SILY 280 revealed features that were in keeping with normal histology. In the group administered with DEX, there was evidence of normal pyramidal cells with deeply stained nuclei, interspersed between degenerating pyramidal cells with pale edges, shrunken and pale staining nuclei. There was also evidence of degenerating granule cells with pale staining pyknotic nuclei. These features are in keeping with neuronal injury.

Examination of sections from groups administered with DEX/SERT, DEX/SILY 140, and DEX/SILY 280 revealed presence of normal looking cells and few degenerating pyramidal/granule cells. The features are in keeping with varying degrees of protection against the development of DEX-induced neuronal injury. In the groups administered with DEX/SERT/SILY 140 and DEX/SERT/SILY 280, the features were in keeping with normal cerebral cortex histology.

Figure 9 shows representative photomicrographs of haematoxylin and eosin stained sections of the dentate gyrus of the mouse hippocampus. Examination of the dentate gyrus region of the hippocampus in the vehicle control group revealed characteristic architecture of the mouse hippocampus with a few large multipolar pyramidal cells of the cornus ammonis 4 region projecting into the concavity of the dentate gyrus. Also observed were well-compacted small granule cells with vesicular nuclei in the ascending and descending arms of the dentate gyrus. Also obvious were astrocytes and microglia, neuronal processes, and nerve cells scattered throughout the molecular layer, that is, lying between the compact zones of the cornus ammonis and dentate gyrus regions. All features are in keeping with normal hippocampal dentate gyrus histology. Examination of the hippocampal dentate gyrus sections of groups fed SERT, SILY 140, and SILY 280 revealed features that were also in keeping with normal

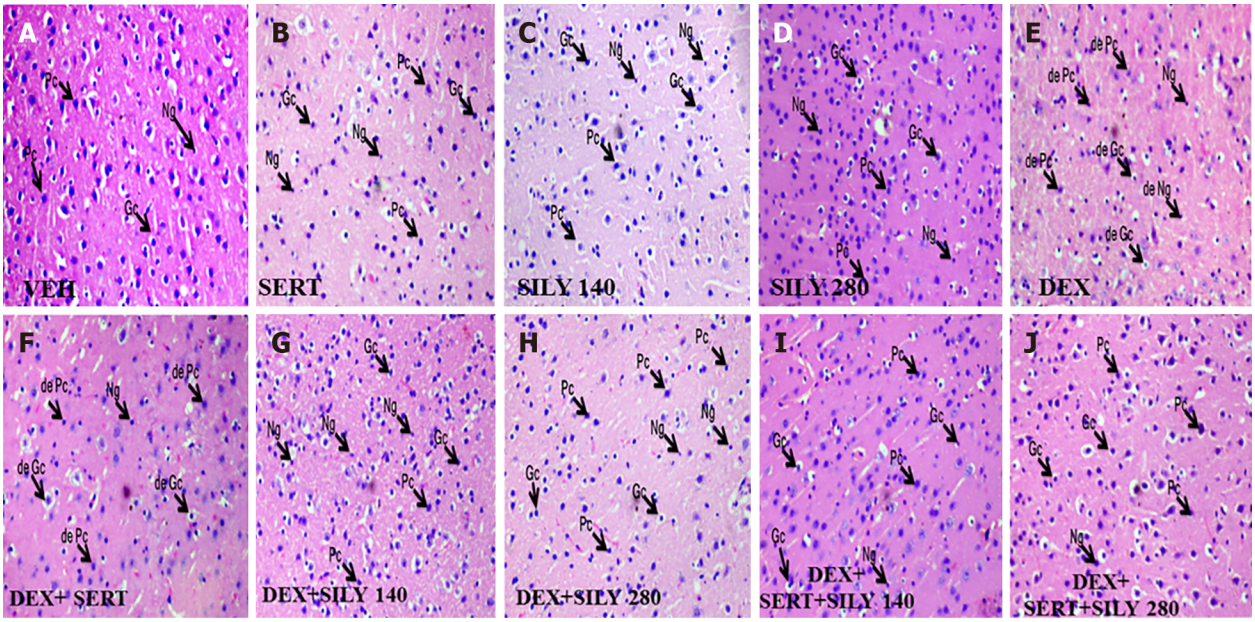

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Figure 8 Effect of silymarin on histomorphology of the cerebral cortex. Photomicrographs show pyramidal cells, granule cells, and neuroglia. A: Vehicle; B: Sertraline C: Silymarin at 140mg/kg of food; D: Silymarin at 280 mg/kg of food; E: Dexamethasone, F: Dexamethasone and sertraline G: Dexamethasone and silymarin at 140; H: Dexamethasone and silymarin at 280; I: Dexamethasone, sertraline and silymarin at 140; J: Dexamethasone, sertraline and silymarin at 280. de-Pc: Degenerating pyramidal cells; de- Gc: Degenerating granule cells; de-Ng: Degenerating neuroglia; Gc: Granule cells; Pc: Pyramidal cells; Ng: Neuroglia. Number of mice per treatment group = 5.

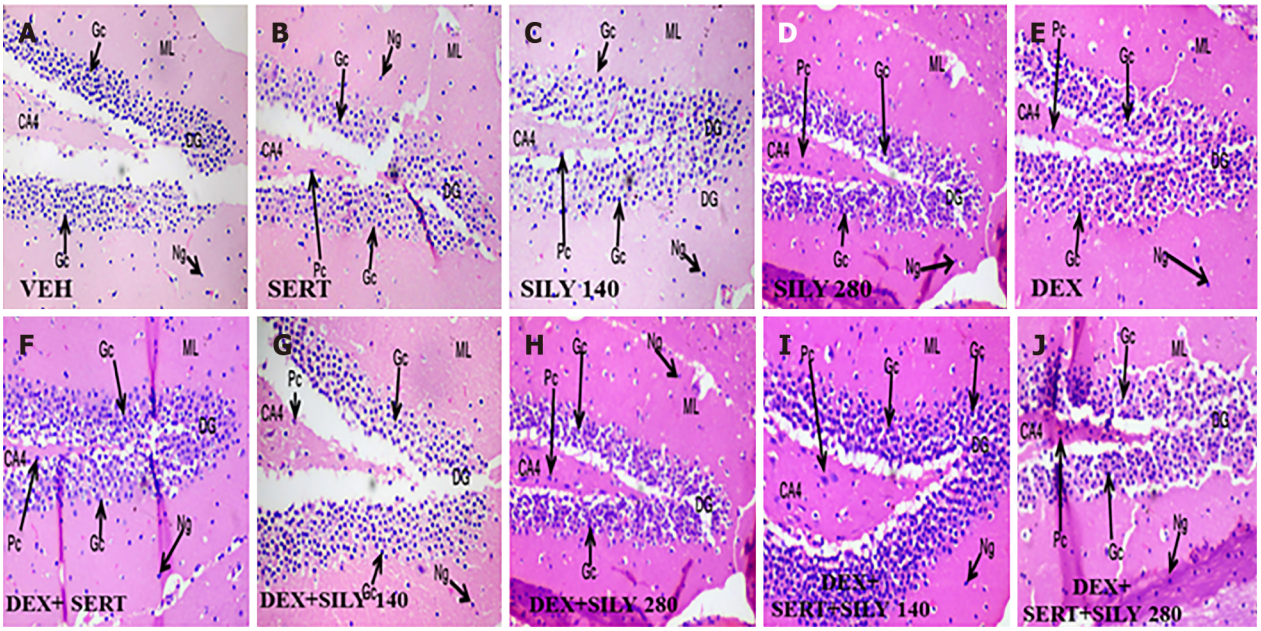

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Figure 9 Effect of silymarin on histomorphology of the dentate gyrus of the hippocampus. Photomicrographs show small pyramidal cells, small granule cells within the dentate gyrus proper, and neuroglia scattered within the molecular layer. A: Vehicle; B: Sertraline; C: Silymarin at 140mg/kg of food; D: Silymarin at 280 mg/kg of food E: Dexamethasone; F: Dexamethasone and sertraline; G: Dexamethasone and silymarin at 140; H: Dexamethasone and silymarin at 280; I: Dexamethasone, sertraline and silymarin at 140; J: Dexamethasone, sertraline and silymarin at 280. Gc: Granule cells; Pc: Pyramidal cells; Ng: Neuroglia; ML: Molecular layer. Number of mice per treatment group = 5.

histology. In the group administered with DEX, there were a few normal small pyramidal neurons interspersed between few degenerating pyramidal cells with pale edges, and there was also a paucity of cells in the molecular layer and loss of compactness of the granule cells in the dentate gyrus. Also observed were a few degenerating granule cells with pale staining nuclei; the features are in keeping with some neuronal injury.

Examination of sections from groups administered with DEX/SERT, DEX/SILY 140, and DEX/SILY 280 revealed presence of normal looking cells and few degenerating granule cells features, which are in keeping with varying degrees of protection against the development of DEX-induced neuronal injury. In the groups administered with DEX/SERT/SILY 140 and DEX/ SERT/SILY 280, the features are in keeping with normal dentate gyrus histology.

DISCUSSION

This study examined the antidepressant-like effects of SILY and SILY/SERT combination in mice to ascertain the role of SILY either alone or as an adjunct to SERT in mitigating DEX-induced behavioural and morphological changes in mice. The results showed that SILY administered alone increased body weight without altering food intake, increased open field locomotor activity, rearing, and grooming, enhanced spatial working memory, and decreased both anxiety-related behaviours and behavioural despair (immobility time in the forced swim and tail suspension tests). This was accompanied by an improvement in antioxidant status, and a decrease in lipid peroxidation, acetylcholinesterase activity, and inflammatory markers. Also, when administered alone or co-administered with SERT, SILY mitigated DEX-induced behavioural, biochemical, and morphological changes in relation to the cerebral cortex and hippocampus.

The impact of body weight and food intake on health, wellbeing, and disease has been reported[59,60]. In this study, administration of DEX was associated with significant weight loss and decreased food intake. While depression is generally associated with excessive weight gain, which has been linked to bingeing on food, according to the *Diagnostic and Statistical Manual of Mental Disorders,* both weight gain and weight loss are symptoms of depression at all ages[2,61]. Similarly, the choice of DEX as a model of depression is centred on its ability to cause dose-dependent weight changes[62,63]. At doses similar to those used in this study, DEX had been associated with weight loss[63], corroborating the results of this study. The results of a study by Poggioli *et al*[64] revealed that chronic administration of DEX was associated with decreased weight gain, which was attributed to its ability to accelerate fatty acid oxidation, and decrease brown adipose tissue thermogenesis and the activity of uncoupling protein-1 mRNA[64]. Weight loss could also be attributed to decreased feed intake which could be secondary to early satiety. The administration of SERT to healthy mice caused a decrease in weight gain without impacting feed intake when compared to mice in the vehicle control group, while increased weight loss was observed in the group of animals administered with SERT with DEX. While there is a dearth of scientific information on the impact of SERT in healthy subjects, it is, however, generally believed that selective serotonin re-uptake inhibitors like SERT are associated with weight gain. The results of a few studies have linked weight gain mainly to long-term use of SERT[65,66]; however, some clinical studies have reported reduced weight gain or weight loss following acute use of SERT in persons with depression[67]. The results of a preclinical study that examined the effect of SERT on body weight parameters in monkeys administered with SERT over an 18 mo period using a placebo-controlled, longitudinal, randomized study design showed that while the body weight and body fat composition of the placebo group increased, a decrease in body weight and fat composition was observed in the SERT treatment group[68]. In the groups of mice fed SILY alone, an increase in weight with no change in food intake was observed compared to mice in the vehicle control group. Also, in mice fed SILY with DEX, a reversal of DEX-induced weight loss was observed. Information from the current literature reveals that the vast majority of studies evaluating the effects of SILY on body weight have administered it in a background of disease or disorder[28,32,69-71]. The results of these studies have shown that administration of SILY could be associated with either weight loss or weight gain[28,32,69-71] depending on the disease model used. This would suggest that the effects of SILY on body weight are mainly modulatory or adaptogenic, having the ability to return the body back to baseline. The administration of SILY with SERT was also associated with a reversal of weight loss due to DEX-induced depressive symptoms, suggesting that compared to SERT, SILY could be beneficial in modulating the effects of SERT on body weight. However, the co-administration of SERT with SILY also in a background of DEX was associated with increased food intake compared to either SILY or SERT.

In this study, neurobehavioural tests revealed that administration of DEX was associated with a decrease in horizontal locomotion, rearing, and grooming behaviour, which is consistent with the observations of Falade *et al*[40]. The chronic unpredictable stress model was also associated with similar neurobehavioural changes[55]. The decrease in locomotor activity, rearing, and grooming is reflective of a central nervous system depressant response to DEX administration. Treatment with SERT was associated with a mitigation of the central depressant effect induced by DEX, although when administered to healthy mice, SERT did not significantly alter horizontal locomotion, rearing, or grooming, which is similar to the response observed by Pereira-Figueiredo *et al*[72]. In healthy mice fed a SILY diet, a central excitatory response was observed at 140 mg/kg. SILY alone or co-administered with SERT reduced the changes in locomotor activity, rearing, and grooming observed in mice administered with DEX alone. The concentration-dependent increase in locomotor activity, rearing, and grooming that occurred in healthy and DEX-treated mice could be linked to its ability to increase brain

levels of serotonin, dopamine, and norepinephrine, neurotransmitters that modulate central excitatory response in the brain[73-76]. Also, the co-administration of SILY with SERT was associated with a significant decrease in line crossing and an increase in grooming, with no significant difference in rearing behaviour compared with mice administered with SERT alone, suggesting that SILY could amplify the effects of SERT.

The neuroprotective effects of SILY have been reported[28,29,77-79] with a number of studies reporting its ability to reverse cognitive deficits and anxiety-related behaviours[79]. In this study, DEX was associated with spatial working memory deficits (Y-maze and radial arm maze) and anxiogenic response in the elevated plus maze paradigm. In past times, cognitive deficits were not considered an important part of depression symptomatology, so little or no attention was paid to cognitive disorders associated with depression. However, in the light of recent knowledge, researchers now know that cognitive symptoms could significantly impact general functioning and quality of life, and risk of recurrence of depression in these individuals[80]. The results of this study demonstrated that while SERT administration was associated with anxiolysis when administered alone or to DEX-treated mice, it showed no nootropic ability in healthy mice. Although it counteracted DEX-induced spatial memory deficits, the results observed with SERT in healthy mice corroborate the report of a study by Siepmann *et al*[81] that showed that in healthy humans, SERT was not associated with cognitive deficits or improvements in cognition. Although SERT reversed memory deficits in DEX-treated mice, studies in humans have reported that a selective serotonin reuptake inhibitor such as SERT was associated with memory loss and anxiety in persons with depression[82]. In groups fed SILY-supplemented diet alone, memory enhancing and anxiolytic effects were observed in both healthy and DEX-treated mice. This effect is similar to that observed by Yön *et al*[79] in diabetic rats. A number of other studies have also reported the ability of SILY to reverse cognitive deficits following scopolamine-induced amnesia[83] or mild traumatic brain injury[84], and these beneficial effects have been linked to its ability to decrease oxidative stress, inflammatory markers, and brain glutamate level, as well as increase antioxidant status and brain-derived neurotrophic factor in rodents[83,84]. Although compared to SERT, the administration of SILY to DEX-treated mice was associated within reversal of memory deficits suggesting that as a sole or replacement therapy it could provide some benefits, large clinical studies are required to confirm these in humans. When co-administered with SERT, memory and anxiolytic effects improved significantly compared to DEX-treated group administered with SERT, and these suggest that SILY could also be beneficial as an adjunct with SERT in depression management.

In this study, administration of SERT or a SILY-supplemented diet was associated with a decrease in immobility time in the behavioural despair paradigm in healthy animals, while DEX caused increased immobility time compared to healthy controls. Several studies have reported that chronic administration of DEX in humans and experimental animals was associated with the development of mood disorders including psychosis and depression[40,85,86]. The ability of DEX to increase immobility time has also been reported by other studies[40,87,88]. However, there is an increasing need for animal models of depression other than the currently available models of behavioural despair (forced swim test and tail suspension test). Animal models such as the one employed in this study supports the glucocorticoid hypothesis of depression[89] and would be valuable in the testing of novel drugs for the management of depression. In this study, chronic DEX administration was associated with weight loss, decreased food intake, locomotor retardation, cognitive deficits, anxiety, and behavioural despair, and a number of these symptoms and signs are necessary for the diagnosis of depression in humans. The mitigation of a number of features by SERT (a conventional antidepressant) supports the face and predictive validity for its possible use as a preliminary method for studying novel pharmacologic agents with possible antidepressant effects. A limitation of this study is our inability to assess plasma or brain glucocorticoid levels. SILY supplementation alone or co-administered with SERT in this study was associated with the reversal of DEX-induced behavioural despair. The antidepressant effects of SILY have been reported especially in studies that used acute restraint stress[76], the chronic unpredictable stress model of depression[90] or posttraumatic stress disorder[91]. In both behavioural despair paradigms, the antidepressant effects of SERT increased significantly with SILY at a concentration of 280 mg/kg of feed, although it decreased at 140 mg/kg of feed, suggesting that high concentrations of SILY could elicit an additive beneficial effect.

The antidepressant, memory enhancing, and anxiolytic effects of SILY have been attributed to its ability to decrease oxidative stress, improve antioxidant status, and increase antiinflammatory markers [76,90]. In this study, dietary SILY supplementation was associated with a mitigation of DEX-induced changes in brain oxidative stress, antioxidant status, and inflammatory markers. It also counteracted DEX-induced increase in acetylcholinesterase activity which could also be responsible for the memory enhancing effects of SILY. When SILY was co-administered with SERT, we observed significant improvements in the oxidant antioxidant balance, and an antiinflammatory response over the effects observed with SERT alone, also reinforcing our opinion that SILY when examined in a rodent model of depression exhibited both adjunctive and sole therapeutic benefits.

Structural and morphological changes have been reported in humans with depression[92,93]. In this study, the administration of DEX resulted in neuronal injury in the cerebral cortex and hippocampal dentate gyrus, two regions of the brain which have been implicated in depression[92-94]. In this study, SERT and SILY-supplemented diet at both concentrations mitigated the structural changes induced by

DEX. The co-administration of SERT with SILY showed marked mitigation of these changes, suggesting that SILY was not only beneficial when administered alone, but it also possibly accentuated the effects of SERT. While our knowledge of the structural and morphological changes in depression and how they impact pathogenesis and treatment are still evolving, it is important to realise that the use of supplements such as SILY that have validated adaptogenic, antioxidant, antiinflammatory, cognitive enhancing, anxiolytic, and neuroprotective effects could be valuable in depression management, although clinical studies and trials would be necessary to verify its usability in humans.

CONCLUSION

The ability of SILY to modulate behaviour, oxidative stress, and neuroinflammation makes it a possible monotherapeutic agent or an adjunct in the management of DEX-induced depression. In this era when clinical management of depression has continued to be challenging, the discovery and application of such an agent are likely to be of benefit in at least a certain subset of patients. The value of an agent such as SILY is likely to rest in the fact that it can employ mechanisms of action that go beyond neurotransmitter modulation.

ARTICLE HIGHLIGHTS

Research background

Depression is a neuropsychiatric disorder that has in recent times become a leading cause of disability and a major contributor to global disease burden and suicide.

Research motivation

There is increasing advocacy for the use of herbal supplements in depression management.

Research objectives

To determine the effect of silymarin dietary supplements alone or in combination with sertraline in a mouse model of depression.

Research methods

Preclinical study.

Research results

Silymarin mitigated dexamethasone-induced central nervous system changes in mice.

Research conclusions

Silymarin could have a place in the management of depression in humans.

Research perspectives

Further studies should be performed to examine the possible effects of silymarin in humans with depression.

FOOTNOTES

Author contributions: Onaolapo AY and Onaolapo OJ conceived and designed the work that led to the submission; Sulaiman H and Olofinnade AT were responsible for the collection and collation of the data; Onaolapo AY and Onaolapo OJ were involved in the analysis of the data, interpretation of the results, and drafting of manuscript; all authors approved the final version of the manuscript.

Institutional animal care and use committee statement: All procedures were conducted in accordance with the approved protocols of the Ladoke Akintola University of Technology and within the provisions for animal care and use prescribed in the scientific procedures on living animals European Council Directive (EU2010/63).

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MINIREVIEWS

Antibiotic residues in milk and milk products: A momentous challenge for the pharmaceutical industry and medicine

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Abstract

Dairy products are nutritious food items that contain various essential nutrients, however, it has been proven that residual antibiotics have contaminated such products. These residues can cause several side effects on human health. They increase antimicrobial resistance against several threatening microorganisms, as well as significant growth in allergenic reactions. Various methods, including heat treatments, have been applied to alleviate and reduce the effect of antibiotic residue level in milk and milk products. Changes in drug levels were not significantly remarkable, obliging researchers to find new approaches to prevent or reduce their risk and limit their complications on human health.

Key Words: Antibiotics residues; Milk products; Bacterial resistance; Antimicrobial drugs; Microorganisms; Health effects

Core Tip: Little information is available regarding the use of antibiotics and their availability in dairy products as residues in Lebanon. Not a lot of care or caution is given to this sector, even though Lebanon's main income, especially in villages, is from cultivated mammals. This article mentions the availability of residual drugs in milk. It includes the different pathways of drugs upon consumption by the mammals, until excretion. As well as its side effect on human health, especially the cause of flora bacteria to become resistant to drugs.

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INTRODUCTION

Dairy products constitute a major category of human diet and are considered one of the more important sources of essential minerals needed for normal body function and growth[1]. The knowledge around the benefits of milk has been growing in the last decades paralleled with a considerable and continuous increase in consumption demand over the years[2]. This necessitates more diligent practices in the production, processing and distribution of milk and dairy products. Understanding the effect of antibiotic use or abuse in protection of milk producing cattle and how this affects the end consumer remains critical to scientists. The notion of antibiotic resistance is gaining more international merit among microbiologists and hence should be addressed when it comes to dairy products and other foods. Mammals can be highly susceptible to many diseases and infections and several microorganisms that are naturally found in nature, could easily become part of their diets, leading to several diseases[3]. Antibiotics are currently used for treating infected animals, similar to humans, by administrating a prescribed dose, targeting certain known microorganisms[3]. Despite the crucial antimicrobial effect of fighting severe diseases, antibiotics still find their way from treated animal into the food products as residues[4]. These residues pose serious health alterations and problems for human health in addition to the aforementioned antibiotic resistance developed by microorganisms[4].

Institutional bodies are imposing stricter regulations by the day and even though many farmers follow a careful protocol in the use of antibiotics while following regulations, residual drugs cannot be totally prevented from passing down into consumers. Herein, we present a review on the significance and importance of testing for antibiotic residues and the challenges associated with the testing techniques. Heat treatment to reduce residual antibiotics concentrations in dairy can be a preliminary approach. Our review can provide important insight on potential effective practices to minimize the detrimental outcome of antibiotic abuse in Lebanon and other countries. Very little is known about such practices in developing and third world countries due to the lack of structured research and regulations. Heavy assessments and examinations should be carried out to ensure the safety of milk products distributed throughout these countries.

MILK AND ITS PRODUCTS

Milk is considered one of the most consumed nutritious food products in the market and along with dairy products remain essential for the human diet since they provide many minerals, vitamins, proteins and sugar, that are much needed for proper growth and development. This highly consumed product is extracted from mammals through their mammary glands, including cattle, goats and sheep [1]. Milk is mostly composed of water (87%), proteins (3%), lactose (4%-5%), fat (3%-4%), minerals (0.8%) and vitamins (0.1%)[5] along with lipids that are usually found in the form of fat globules and are considered carriers of fat-soluble vitamins, as well as flavor enhancers[5].

The demand for milk and milk products is widely discussed in political, agricultural, and economic meetings including the EU Agricultural Outlook conference. The consensus is that dairy demand will drastically increase by 2030, given the fact that milk is an affordable source of proteins and nutrients that is commonly linked to healthier life styles[2]. In one study targeting Middle Eastern and North African populations, associated higher milk consumption with increased population growth in which a 1.0% increase in population caused a 1.3% increase in consumption[6].

All dairy products share the first processing step of milk fermentation for 1-3 d at room temperature (15 °C-25 °C)[7]. These products could also be generally categorized into two groups; products considered new or those later discovered from practices in the industrial field of production and development known as "Modern Dairy Products", and products derived from ancient practices mostly used in small-scale societies and called "Traditional Dairy Products". Some of the modern products

include milk (total, skimmed, and semi-skimmed), butter, cream (fresh, double, and ice), and cheese (hard, semi-hard, soft, and fresh). Some traditional products, mainly carry Arabic names derived from different Arabic countries, including laban, labneh, ayran, kishk, and some different types of cheese such as akawieh, mish cheese, dominate cheese, and haloumi[7].

Like humans, animals are highly susceptible to many different diseases. They could get sick due to exposure to different microorganisms if protective measures are not in place. Despite Lebanon's small size, it is known that there is a fair proportion of the population that relies on milk and dairy products as a source of income[3]. Microbial infections and other diseases present major challenges to indigenous local farmers as well large industrial producers. Furthermore, mastitis is the most common fatal disease in dairy cattle which results from bacterial infection from the external environment, such as contaminations from non-sterile milking equipment, milking personnel, or dirty stalls[8].

Mastitis is one of many other reasons why antimicrobial use has gained attention in dairy farms[9]. Hence, antimicrobials are introduced to cows when the animal is sick to assure the wellbeing of both the cattle and humans down the food chain[10].

ANTIBIOTICS

Antibiotic treatment in animals is generally used either to help and protect the animals' welfare, or in some cases, used for the farmers' benefit to increase the animals' weight beyond the normal[11]. In the case of the animal's wellbeing, antibiotics are used in one of two fashions; either prophylactically, through the injection of medications or vaccines to prevent any possible illness, especially severe ones, or therapeutically to aid in infighting a certain illness or organism that is considered a health threat[11].

Antibiotics used for animals should be limited as much as possible for obvious reasons including serious accumulation in higher consumers. They are regulated within certain "safe" levels to prevent side effects, such as the development of antibiotic resistance in animals. For this reason, certain parties should be involved to ensure the maintenance of the efficiency and safety use of veterinary antibiotics [11].

Different medicines with antimicrobial activity are used depending on their target bacteria and mode of action. Some happen to have similar characteristics and act on the same pathogens. Others are used over a wide range, effective on more than one microorganism[12]. Penicillin is a commonly used drug for humans and several animals and is considered one of the first discovered and used drugs. It is a medication used against many types of microorganisms. Similar to penicillin, cephalosporins are a group of bactericidal molecules that also possess antibiotic properties[12]. Tetracycline is another common drug used for different purposes, mostly administrated when penicillin cannot be used[12]. Aminoglycoside is a drug used therapeutically and prophylactically; it works against Gram-negative bacterial infections[7]. Streptomycin is a common type of aminoglycoside used in dairy cows[12]. Moreover, potentiated sulphonamides are a combination of both antibiotics, sulphonamides, and trimethoprim, used for the treatment of different bacterial infections[12].

Nonetheless, if not controlled, microorganisms can make their way to the human body through food, human-animal contact, or the environment. When bacteria are successfully targeted, antibiotics would then be discharged out of the animal's body. However, antibiotics are not completely released from the animal and a certain amount of these drugs remains concentrated in some tissues or products (such as milk) that will eventually be consumed by the human[11].

Antibiotic abuse

Antibiotic misuse, also called antibiotic abuse, is the inappropriate use of antibiotics in levels higher than required. In many areas, there is no proper inspection for the use of drugs, especially in third world countries. For instance, many local farms in Lebanon within different villages are left with no auditing or oversight. Unlike other countries, farmers mostly do not need a prescription and are most likely not supervised by a vet upon antibiotic administration, making it more likely to either use drugs in overdosage or for the inappropriate target. This lack of knowledge regarding antibiotics' use and their mechanisms could cause negative consequences for both, animals and humans[13].

Other than AMR, residues could cause disorders of the intestinal flora, severe allergic reactions, and hypersensitivity[4].

Antibiotics absorption, distribution, and excretion

Upon authorized use of antimicrobials within the respected doses, and its administration to the animal, a major part of this drug is detoxified and excreted. Since antibiotics could easily transfer from the mammary gland to the milk, it is thus the main cause for residues presence in milk and its products[14]. Antibiotics progress through the animal's body after their introduction and are absorbed and distributed into different parts of the body. They are then eliminated by being metabolized to perform their normal function or secreted out of the body.

Several studies have been aimed at understanding the metabolism and retention of antibiotics in both animals and consumers. One study assessed the distribution of antibiotics after the intramammary

infusion of different antibiotics, and physical properties were examined instead of the molecular configuration of the antibiotics[15]. It was concluded that absorption of drugs is highly dependent on the lipid-solubility of the introduced drug and on the dissociation constant (pK_a). The pK_a determines the concentration of the undissociated form of the drug in milk, as well as the rate of the drug passage from milk to the blood. The degree of lipid-solubility is determined by comparing the lipid-to-water partition coefficient ($Ko_{/w}$). The higher the $Ko_{/w}$, the faster the drug is absorbed. When comparing both factors, it seemed that the rate of fusion from milk to drug was dependent on the degree of lipid-solubility of each drug, thus pK_a became the main factor affecting absorption of drug from the udder[16]. In addition, it also appeared that some antibiotics could bind to proteins which will eventually affect the rate of absorption of the drugs[15].

Another study involved four lactating goats that were injected with radioactive-labeled antibiotics by intramammary infusions. After milk samples were collected, antibiotic concentrations were evaluated and collected from several dairy products; skim milk, whey, cream and casein were compared to whole milk, using either microbiological or radiochemical assay methods[15]. In the case of cream, concentrations were shown to be inversely proportional to the concentration of the drug in the product[17]. The percentage of drugs distributed in the different milk components differs depending on the type of antimicrobial used[16]. Whereas, when comparing residues within the same product, high concentrations showed lower residues with a percentage close to that of water in cream, while low concentrations of certain drugs displayed a higher level in the product[17]. The concentration of drugs in casein was highly dependent on the percentage of antibiotics consumed. In the case of skim milk, concentrations depended on the interaction of drugs with the proteins. Other studies showed that the concentration of drugs in casein is usually higher than in whey since proteins in casein are largely higher than in whey, thus antibiotics would concentrate with the proteins[17].

Yet, other studies showed that after intramammary injection of penicillin into one of the quarters of the cow, the antibiotic was detected in the other 3 quarters[18]. It could be concluded that the drug is capable of interstitial migration from one area to another. Moreover, a study was performed to analyze the absorption, distribution, and excretion of two drugs; penicillin G and dihydrostreptomycin, a type of aminoglycoside[18]. Six cows free of clinical mastitis were treated each by injecting 100000 units of penicillin G and 100 mg of dihydrostreptomycin into three quarters (1, 2, and 4). This treatment was repeated twice for each cow after a 2-wk rest. Results showed that in the case of two quarters treatments, penicillin G was found in untreated quarters after 8 h' rest[18]. There was also a diffusion of the drug into untreated quarters in the case of dihydrostreptomycin. Five cows out of six were found to have drug residues in untreated quarters[8,18]. Blood, milk, and urine samples were collected from each cow and results revealed the presence of both drugs in the three different samples; the highest level was detected in the blood, followed by the milk, and urine. This indicates that drug crossover from treated to untreated quarters is not only directly through the blood; there could be another pathway or mechanic for this distribution[8,18].

In the case of antibiotic excretion, the percentage of drugs released from the cow during milking clearly decreases as the time of administration is longer. Antibiotics are excreted partly which would still hold complications during manufacturing and later for human health after consumption. Depending on the type of antibiotics, 70%-80% of the drug is found in the first milking after administration[19]. That explains why first milking should be discarded when cattle are treated with antibiotics.

Bacterial resistance

The misuse and overuse of antimicrobial agents is the main reason for the increase in antimicrobial resistance (AMR). The concept has evolved quickly and is a big concern worldwide since it could impact health in general and for decades[20]. Bacteria have remarkable genetic flexibility that allows them to respond to any environmental threats. Constant exposure of the drug to certain microorganisms could lead to reduced effectiveness due to changes developed by the organism itself. A bacterium can acquire resistance by either modifying its DNA during cell replication referred to as mutation, or by inserting the organism's gene into its own, becoming part of the bacteria's genetic material thus becoming resistant to the drug. Therefore, upon introduction of the drug again, only non-resistant organisms are affected while resistant bacteria remain unaffected and preferentially proliferate. Each organism acquires resistance differently, depending on its type. For instance, the resistance mechanisms of β-lactams differ depending on the type of bacteria. For Gram-negative, it is modified in a way to produce β-lactamases, while in Gram-positive bacteria, it is achieved by resistance by modifying its target site; the penicillin-binding proteins[20].

To provide a complete classification of antibiotic resistance, they can be categorized depending on their biochemical pathway related to the drug[20]. Modifying the antibiotic molecule can be done by either producing enzymes that inactivate the drug, or by destroying the molecule itself, thus preventing the drug from interacting with its target. For example, aminoglycoside modifying enzymes can modify the hydroxyl or amino group of the aminoglycoside drug, reducing or eliminating the drug's activity. Another mechanism is preventing the reach of the antibiotic to its target, achieved by reducing the penetration of the antimicrobial compound[20]. The third mechanism includes the modification of the binding sites.

Drug identification

Milk should be tested, before collection and delivery to the truck, to ensure its safe use. It is imperative to test for antimicrobial residues before processing, to ensure its compliance to accepted doses[21]. If antibiotics tested exceed the maximum residue limit (MRL), then milk collected should be disposed, and not used for consumption or product manufacturing[14]. There are several techniques available for residual drug identification, yet liquid chromatography has demonstrated to be the most generally effective, definite and of maximum sensitivity. Milk samples are usually found to be rich in more than one type of antibiotic, thus tests are usually done to identify the type of each drug, and its concentration [21]. One group of researchers analyzed a set of milk samples to identify the presence of several antibiotics using the liquid chromatography technique. The antibiotics investigated were cloxacillin, dihydrostreptomycin, tetracycline, oxytetracycline, chlortetracycline, chloramphenicol, neomycin, novobiocin, bacitracin, erythromycin, oleandomycin, ampicillin, streptomycin, and oxacillin[21]. Different analytical grade reagents were used to extract each antibiotic present, such as hydrochloric acid, citric acid, ammonium chloride, and others. The solvents used were as follows: (1) Chloroform-acetone-impregnation liquid (5:5:2); (2) ethanol-water-ammonia (8:1:1); (3) methanol-chloroform (9:1); (4) methanol-acetone (3:2); and (5) methanol-ammonium chloride (3%). The three adsorbents used to coat the thin layer plates were kieselguhr F254, silica gel 6o, and cellulose. The organisms used for testing were *Bacillus ceurus*, *Bacillus subtilis* (*B. subtili*), *Micrococcus flavus* and *Sarcina lutea*. The approach involved 6 different plates of either a control or the mentioned microorganisms being targeted by each drug[21]. Table 1 represents the scheme followed to implement the chromatography technique.

Furthermore, Table 2 shows the R_f values of the antibiotics on different plates. This is indicative that the minimum concentrations of such antibiotic are still detected by liquid chromatography and can be identified using this method. Penicillin was not tested since its presence could be determined by using the *B. calidolactis* test. It was eliminated or removed from the samples by adding an enzyme; penicillinase, which would cause its degradation. Other techniques that could be used for the identification of antibiotic residues in the milk include paper chromatography, high-tension electrophoresis, gas chromatography, mass spectrometer and thin-layer chromatography. Some of these techniques were shown to be of little and inefficient use because of their need for highly concentrated solutions with antibiotics or only being efficient for certain drugs[21].

Yet another technique that could be used is known as the Screening Test for Antibiotic Resides (STAR) protocol. STAR protocol is a five-plate test (FPT), which involves agar diffusion for the identification of antibiotics in food. It is a qualitative screening test used to detect bacterial growth inhibitors. Five agar plates are used to detect antibiotics using sensitive known bacterial strains. The formation of inhibition zones would prove the presence of the antibacterial substance[22].

Near-infrared absorption (NIR) spectroscopy is also used for residue identification. Using a detector, this technique can analyze and record different wavelengths of each substance present in the solution. The intensity of each wavelength computed is equivalent to the concentration of the substance or drug. A study was conducted using the NIR technique for different types of antibiotics, it was proven to be fast and accurate, thus proving the method's great potential[23].

As a result, the chromatographic technique could be considered as the most efficient one in detecting the highest number of antibiotic residues because of its higher sensitivity and specificity and higher quantification capability (Sachi *et al*[14]).

Heat treatment

During processing, the milk is exposed to different thermal, chemical, or mechanical shocks. This includes boiling at high temperatures, the addition of acidic substances, and reduction in water activity, cooling or freezing, drying, or evaporation. These changes could alter the product's characteristics[24].

A study was performed to assess the effect of high temperature and pH on tetracycline and azithromycin concentrations. *B. subtilis* was used to test the presence of these drugs in 13 milk samples. The results showed that high concentrations of residues were present in the samples; high enough to inhibit the growth of *B. subtili* and to kill the microflora culture during milk fermentation. The effect of heat treatments on the degradation or reduction of residues varied amongst different temperature treatments and pH[25].

In the case of azithromycin, a significant decrease of the azithromycin constant κ was observed at 70 °C after 3 h incubation, followed by a higher reduction during 24 h incubation (Table 3). Similarly, a high decrease of constant κ was also observed at 100 °C after incubation[25]. On the other hand, a significant increase in the tetracycline constant κ was observed during incubations. Tetracycline constant κ showed a fast decline at 70 °C and 100 °C after 24 h incubation (Table 4). Results showed that the stability of both drugs, tetracycline, and azithromycin, is highly dependent on temperature[25]. Using *B. subtili* culture, no inhibition areas were observed, proving that azithromycin lost its antimicrobial activity after treatments of 70 °C and 100 °C for 24 h. In the case of tetracycline, the Inhibition zone was reduced but not inhibited upon the increase of temperature[25].

Table 1 Chromatographic scheme

Thin-layer plate	Adsorbent	Amount of sample applicated in µL	Solvent	Test organism
1	Kieselguhr	30	A	*B. cereus*
2	Silica gel	30	B	*M. flavus*
3	Silica gel	30	C	*S. lutea*
4	Silica gel	30	C	*B. subtilis*
5	Silica gel	30	D	*M. flavus*
6	Cellulose	10	E	*B. cereus*

B. cereus: *Bacillus cereus*; *M. flavus*: *Micrococcus flavus*; *S. lutea*: *Sarcina lutea*; *B. subtilis*: *Bacillus subtilis*.

Table 2 R_f values of antibiotics on the different plates

Antibiotic	Plate					
	1	2	3	4	5	6
Cloxacillin	-	-	0.70	0.70	-	0
Dihydrostreptomycin	-	-	-	-	-	0.40
Tetracycline	0.35	0	0	0	0	-
Oxytetracycline	0.20	0	0	0	0	-
Chlortetracycline	0.60	0	0	0	0	-
Chloramphenicol	0	0.70	0.65	0.65	0.80	-
Neomycin	-	-	-	-	-	0
Novobiocin	-	0.80	0.80	0.80	0.80	-
Bacitracin	-	0.45	-	-	-	-
Erythromycin	0.20	0.70	0.35	0.35	0.25	-
Oleandomycin	0.20	0.70	0.35	-	0.25	-
Ampicillin	0.35	-	0.50	-	-	-
Streptomycin	-	-	-	-	-	0.50
Oxacillin	0	-	0.60	0.60	-	-

CONCLUSION

Milk contains many important nutrients that are required daily. Milk components take part in the body's metabolism by providing essential amino acids, vitamins, minerals, and fatty acids. The presence of drug residues in dairy products is harmful and not acceptable to many individuals or food industries. The use of antibiotics should be controlled and handled under the control of specialists. Antibiotics are absorbed by animal tissues and later distributed into different fluids and tissues. A high concentration is later excreted after a few hours, but a significant amount would still be found concentrated in different parts, including milk and meat. Heating treatments do not cause significant residual reduction among all drugs. High temperatures are effective against azithromycin, where it causes a drastic reduction, while it is less effective against tetracycline. There are no techniques effective on all drugs and that significantly decrease antibiotic residue level. Antibiotic usage in animals cannot be completely banned since their absence could be harmful to both the animal and human health. More research should be conducted to identify new programs that could possibly reduce and control their usage or their concentration in milk and milk products.

Table 3 Azithromycin degradation constant rate k values at different temperatures[10]

Time in h	4 °C	37 °C	70 °C	100 °C
1	1000 ± 8[e]	50 ± 1[a]	50 ± 2[a]	200 ± 3[a]
3	333 ± 3[d]	966 ± 9[d]	4033 ± 22[e]	3900 ± 18[e]
6	250 ± 2[c]	933 ± 8[d]	2000 ± 15[d]	2033 ± 16[d]
12	116 ± 2[b]	516 ± 7[c]	1025 ± 8[c]	1000 ± 6[c]
24	62 ± 2[a]	270 ± 4[b]	537 ± 5[b]	533 ± 4[b]

[a]Different letters indicate statistically significant differences at $P < 0.05$ according to a Turkey's honest significant difference *post hoc test*. Values are mean ± standard error of triplicate samples.
[b]Different letters indicate statistically significant differences at $P < 0.05$ according to a Turkey's honest significant difference *post hoc test*. Values are mean ± standard error of triplicate samples.
[c]Different letters indicate statistically significant differences at $P < 0.05$ according to a Turkey's honest significant difference *post hoc test*. Values are mean ± standard error of triplicate samples.
[d]Different letters indicate statistically significant differences at $P < 0.05$ according to a Turkey's honest significant difference *post hoc test*. Values are mean ± standard error of triplicate samples.
[e]Different letters indicate statistically significant differences at $P < 0.05$ according to a Turkey's honest significant difference *post hoc test*. Values are mean ± standard error of triplicate samples.

Table 4 Tetracycline degradation constant rate k values at different temperatures[10]

Time in h	4 °C	37 °C	70 °C	100 °C
1	20 ± 4[a]	50 ± 6[a]	30 ± 6[a]	60 ± 15[a]
3	256 ± 32[e]	400 ± 46[e]	630 ± 81[e]	860 ± 61[e]
6	163 ± 17[cd]	290± 23[cd]	360 ± 17[cd]	510 ± 46[d]
12	116 ± 26[c]	225 ± 12[c]	300 ± 26[c]	340 ± 32[c]
24	50 ± 8[b]	135 ± 20[b]	160 ±18[b]	160 ± 02[b]

[a]Different letters indicate statistically significant differences at $P < 0.05$ according to a Turkey's honest significant difference *post hoc test*. Values are mean ± standard deviation of triplicate samples.
[b]Different letters indicate statistically significant differences at $P < 0.05$ according to a Turkey's honest significant difference *post hoc test*. Values are mean ± standard deviation of triplicate samples.
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[e]Different letters indicate statistically significant differences at $P < 0.05$ according to a Turkey's honest significant difference *post hoc test*. Values are mean ± standard deviation of triplicate samples.

FOOTNOTES

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