



Phytochemical Components Analysis of Bitter Melon Leaves Extract (*Momordica charantia* Linn) Using LC-HRMS and Study the Anti-depressant Activity Possible Effect on Brain BDNF

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Abstract

Depression is a psychiatric disorder that affects mood and physical health and contributes significantly to the global burden of disease. Various molecular mechanisms in the brain are associated with the cause of symptoms and severity of depression. Brain-derived neurotrophic factor (BDNF) is a neurotrophin involved in depressive disorders. Many preclinical and clinical studies provide evidence that BDNF is involved in behavioral phenomena associated with depression. Herbal medicine can be used as an alternative for the treatment of depression. Previous studies have reported that bitter melon leaves (*Momordica charantia* Linn) have antidepressant activity *in vivo* by observing the behavior of test animals. However, until now, there has been no report on the active compounds in bitter melon leaves extract responsible for antidepressants and their effects on BDNF levels. Based on this, this study aims to determine what active compounds are contained in bitter melon leaves extract that is suspected of having antidepressant activity *in vivo* tests through changes in the behavior of test animals and tracking their effects on changes in BDNF levels. This research methodology commences with the analysis of the compound composition of 80% ethanol extract from bitter melon leaves via LC-HRMS. Subsequently, the antidepressant efficacy of the ethanol extract is evaluated at dosages of 200 and 400 mg/kg BW *in vivo*, utilizing the Chronic Unpredictable Mild Stress (CUMS) model in mouse subjects. Behavioral outcomes are assessed by measuring the duration of feeding in the Novelty Suppressed Feeding (NSF) Test, followed by an examination of the effects on BDNF levels in the hippocampus using an ELISA kit. Research shows that in the 80% ethanol extract of bitter melon leaves through LC-HRMS test, 7 compounds were found that had peaked with relative abundance greater than 1%, namely α,α -Trehalose, Stearamide, 1-Stearoylglycerol, 2,2'-Methylenebis (4-methyl-6-tert-butylphenol), 16-Hydroxyhexadecanoic acid, Oleamide, and Corchorifatty acid F. The administration of 80% ethanol extract from bitter melon leaves with doses of 200 and 400 mg/kgBW *in vivo* enhanced behavioral improvement by increasing feeding time and elevating BDNF levels in the hippocampus. The study's results indicate that bitter melon leaves contain active chemicals that exhibit effective antidepressant properties by inducing behavioral changes and enhancing BDNF levels in the hippocampus.

Keywords: Depression; BDNF; Bitter Melon; LC-HRMS; *Momordica Charantia* Linn

Abbreviation

BDNF: Brain Derived Neurotrophic Factor; CUMS: Chronic Unpredictable Mild Stress; ELISA: Enzyme Linked Immunosorbent Assay; GABA: Gamma Aminobutyric Acid; HPA: Hypothalamic Pituitary Adrenal; LC-HRM: Liquid Chromatography Orbitrap High Resolution Mass Spectrometry; NSF: Novelty Suppressed Feeding; SSRIs: Selective Serotonin Reuptake Inhibitors; UAE: Ultrasonic Assisted Extraction; 5-HT: 5-hydroxytryptamine; VTA: Ventral Tegmental Area

Introduction

Depression is a psychological condition defined as a mood disorder identified by sadness, anhedonia, guilt, low self-esteem, alterations in sleep and appetite, diminished concentration, and fatigue, which may become chronic and recurrent, impairing an individual's ability to perform daily activities effectively [1,2].

Prevalence rates vary by age, peaking in older age or adulthood (above 7.5% among women aged 55–74 years and above 5.5% among men). Depression also occurs in children and adolescents under the age of 15 but at a lower rate than in older age groups [3]. It is estimated that 3.8% of the population, or around 280 million people in the world, experience depression, and more than 700,000 people experience suicide. Suicide is the fourth leading cause of death among 15-29 year olds [4]. The most commonly prescribed medications for the treatment of depression are selective serotonin reuptake inhibitors (SSRIs); on the other hand, they can cause sedation, fatigue, gastrointestinal disorders, agitation, and insomnia. Although SSRI antidepressant drugs have been proven to be effective, they are still not optimal due to the high levels of depression and cumulative remission rate is only 30% [5]. Approximately eighty per cent of the global population depends on herbal medicinal products as their main source of health care [6]. Some herbs have been extensively studied and have shown benefits comparable to standard antidepressant medications, with positive data showing fewer side effects compared to conventional medications [7]. Research in herbal psychopharmacology has increased rapidly over the past few decades. However, caution should be exercised when interpreting the results, as many studies have not been replicated [8]. Neuroprotective compounds contained in herbs are interesting to explore as adjuncts to drugs or therapies. The content of active molecules and effectiveness testing are important [9]. To determine the characteristics or content of active molecules of

an herb, high-resolution mass spectrometry (HRMS) can be used, which refers to the highest precision technique for measuring a molecule's mass-to-charge (m/z) ratio. This technique uses various analytical tools to achieve accurate measurements [10]. Bitter melon leaves (*M. charantia* Linn.) have antidepressant effects that depend on serotonergic (5-HT₂ receptors), noradrenergic (α 1- and 2-adrenoceptors), dopaminergic (D₂ receptors), and cholinergic systems and muscarinic anxiolytic effects [11]. The antidepressant effect of methanol extract of bitter melon leaves (*M. charantia* Linn.) at 300 mg/kg BW is comparable to imipramine at a 5 mg/kg BW dose in male Swiss albino mice [12]. Synaptic serotonin increases neuroprotective proteins like BDNF. Depression medication normalises BDNF levels. Depression remission occurs when BDNF and neuroplasticity rise [13].

Numerous animal studies investigating antidepressants employ the Chronic Unpredictable Mild Stress (CUMS) method to induce depressive-like states. CUMS induces abnormalities across somatic, physiological, neurological, biochemical, and behavioural domains by disrupting homeostasis. The vulnerability to anhedonia induced by CUMS is linked to reduced gene expression and alterations in dopaminergic neurones within the ventral tegmental area (VTA) and the hypothalamic-pituitary-adrenal (HPA) axis [14-16]. Behavioural tests have been created to validate and substantiate cognition, emotion, and psychopathology theories. Behavioural assessments for anxiety and depression frequently employ similar methodologies, one of which is the NSF test [17]. The NSF is an assessment that entails regulating or suppressing eating behaviour. A reduction in eating behaviour constitutes a manifestation of stress and may lead to diminished levels of phospho-mTOR in the prefrontal cortex, a signalling molecule linked to acute depression [18,19]. BDNF stimulates mTOR to modulate glur1 expression required for memory formation and is associated with depression [20]. Previous studies have shown that the activity of bitter melon leaves (*M. charantia* Linn.) in depressive disorders is still limited. Based on the current evidence, it is important to research the content of bitter melon leaves as an antiperspirant agent and explore its effects on behavioral changes and BDNF levels.

Materials and Methods

Animals

The experimental animals used were Balb/c males, 2-3 months old, weighing 20-30 grams. They were obtained from the Depart-

ment of Pharmacology, Gadjah Mada University, Yogyakarta. The experimental animals were stored and treated in the Pharmacology Department, Faculty of Pharmacy, Gadjah Mada University. The research began after obtaining ethical permission from the Ethics Commission of the Faculty of Medicine-Public Health and Nursing, Gadjah Mada University (MECHC).

Chemicals

Methanol (MeOH), acetonitrile (ACN), and water of liquid chromatography-mass spectrometry (LC-MS) grade were obtained from Fisher Scientific (Hampton, NH, USA). Formic acid, hydrochloric acid (HCl), and methanol of HPLC grade were procured from Merck (Darmstadt, Germany). The solution of positive ions comprises caffeine, MRFA, and Ultramark 1621. Radioimmuno-precipitation assay (RIPA) buffer with protease inhibitors (Santa Cruz Biotechnology inc., Tokyo, Japan), BDNF (Brain Derived Neurotrophic Factor) ELISA Kit (EM0020-96well) (FINETEST) (Wuhan Fine Biotech Co., Ltd)

Plant collection

Bitter melon leaves simplicia was purchased at BALITRO Bogor, West Java. The bitter melon leaves used as test samples were determined at the Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University.

Plant extraction

Bitter melon leaves extract is made using the Ultrasonic Assisted Extraction (UAE) method, a modified maceration method that adds ultrasound (a signal with a high frequency of 20 kHz) for 1 hour. The solvent used is 80% ethanol with a material-to-solvent ratio (1:7). The macerate is then filtered and optimized in a water bath at a temperature below 80°C.

Metabolite profiling using LC-HRMS

The metabolite content of bitter melon leaves extract was assessed by extracting raw data from a total ion chromatogram generated in an LC-HRMS metabolomic study. We employed a Thermo Scientific™ (USA) Acclaim™ PepMap™ 100 C18 HPLC analytical column, characterized by a length of 150 mm, an internal diameter of 1 mm, and a particle size of 3 μm . The volume of injection given was three μl . Measurements were carried out using mass spectrometry, performed in both negative and positive ionization modes, utilizing full MS/dd-MS2. The sheath rate of gas flow was calibrated to 15 arbitrary units (AU), and the additional gas flow rate was set at 5

AU. The capillary temperature was held at 300°C, while the spray voltage was established at 4.00 kV. The scanning range spanned from 150 to 2000 m/z, with a resolution of 140,000 for carrying out MS and 17,500 for dd-MS2. The study utilized the Compound Discoverer® software created by Thermo Scientific (USA). The analysis included all data, encompassing blanks with methanol. The results were subsequently filtered according to the relative abundance of metabolite compounds.

Chronic unpredictable mild stress (CUMS)

Stress induction was carried out according to the Chronic unpredictable mild stress (CUMS) method [21-24]. Experimental animals were exposed to stressors for 4 weeks according to a random schedule to avoid habituation. Control experimental animals were left undisturbed in their cages except for treatment tests. Exposure to stress includes tilting the drum at 45° for 24 hours, fasting from eating for 24 hours, emptying the drinking bottle for 4 hours, clamping the tip of the tail for 2 minutes, swimming in water at 30° for 20 minutes, fasting from drinking for 24 hours, and hanging the mouse's tail for 30 minutes. The CUMS model is regarded as the preeminent animal model of depression, effectively simulating the human state. Nevertheless, this model is vulnerable to slight design alterations [15,16].

Antidepressant Activity test

Anti-depression activities utilize the Novelty-Suppressed Feeding Test (NSF). The NSF serves as a commonly employed behavioural paradigm for evaluating depression-like behaviour in experimental animals post-fasting. The assessment analyzes the conflict between consumption and anxiety related to unfamiliar environments. The mice were acclimatized in the testing room overnight after a 24-hour fasting period for the experimental subjects. A platform with food pellets was placed at the centre of a square open box with dimensions of 100 × 10 × 10 cm (length × width × height). Each animal was positioned in the test apparatus, maintaining uniform orientation and alignment toward the platform. The time taken by each animal to consume the food pellet was recorded over 5 minutes; during this assessment, non-eating behaviours, such as touching and kissing, were excluded from the definition of eating [21,25,26].

Measurement of BDNF

According to the manufacturer's instructions, BDNF concentrations in the hippocampus were measured utilizing an ELISA

kit (Wuhan Fine Biotech Co., Ltd). Proteins, or standards, are employed to determine BDNF levels. Samples were first treated with BDNF antibody and subsequently incubated for 60 minutes with a streptavidin-horseradish peroxidase conjugate reagent. A substrate was then introduced to terminate the reagent, and absorbance at 450 nm was measured using a multi-detection microplate scanner (Powerscan_1-42, HT, Dainippon Pharmaceutical, Japan).

Results and Discussion

Phytochemical analysis employing liquid chromatography-orbitrap high-resolution mass spectrometry

Bitter melon leaves (*M. charantia* Linn.) contain steroids, triterpenoids, alkaloids, flavonoids, tannins, amino acids, reducing

sugars, tannins, and saponins [12]. Bitter melon leaves exhibit antidepressant effects at 50-400 mg/kg body weight [11]. To the knowledge of researchers, until now, no research has been carried out to find out what active compounds contained in bitter melon leaves have antidepressant activity, so in this study, the compounds contained in bitter melon leaves extract were identified using LC-HRMS. Analysis of plant metabolites found in bitter melon leaves extract in positive and negative ionization modes yielded seven compounds from three updated online databases: Predicted Composition, mz Cloud Search, and Chem Spider Search. Filtering the metabolite compounds detected in each database based on their name and relative abundance produces chromatograms and metabolite compounds presented in figure 1 and table 1.

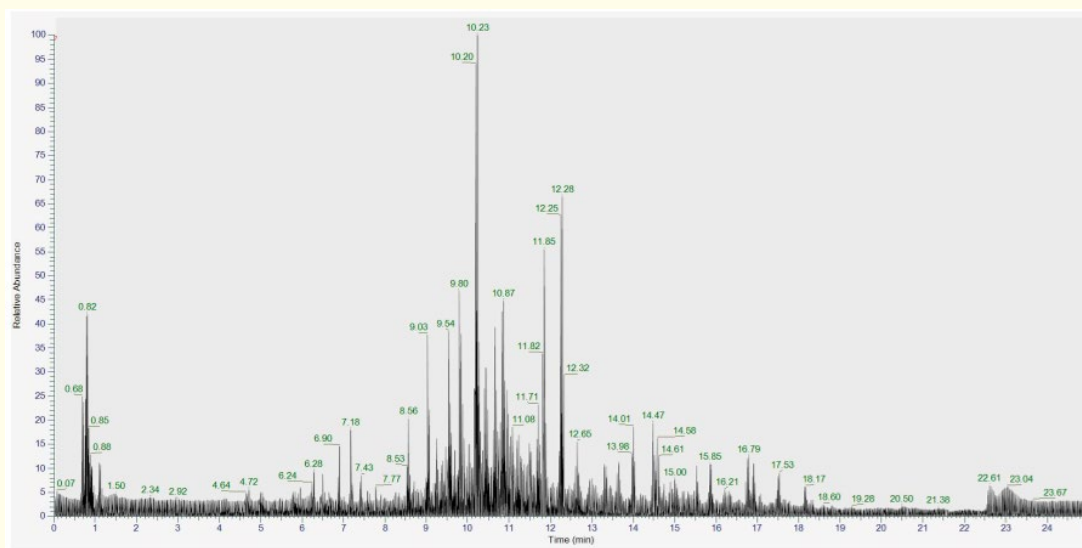


Figure 1: The total ion chromatogram of an ethanolic extract bitter melon leaves was studied using LC-HRMS with a retention period of 0.00-25.00 minutes. Only peaks with a relative abundance greater than 1% were selected. LC-HRMS stands for liquid chromatography-orbitrap high-resolution mass spectrometry.

Metabolite name	Formula	Retention time (min)	Molecular weight	Area (Max.)	Relative abundance (%)	mzCloud Best Match
α,α -Trehalose	C12 H22 O11	0,77	342,12	1226514724,64	5,51	92,5
Stearamide	C18 H37 N O	15,87	283,29	624311573,75	2,80	99,5
1-Stearoylglycerol	C21 H42 O4	15,53	358,31	547075815,88	2,46	95,9
2,2'-Methylenebis(4-methyl-6-tert-butylphenol)	C23 H32 O2	15,88	340,24	368677823,09	1,66	98,6

16-Hydroxyhexadecanoic acid	C16 H32 O3	13,95	272,23	281177363,70	1,26	93,1
Oleamide	C18 H35 N O	14,91	281,27	261719624,99	1,18	98,6
Corchorifatty acid F	C18 H32 O5	8,60	328,22	223085934,65	1,00	97,7

Table 1: The metabolite compound content of bitter melon leaves extract was detected using LC-HRMS.

Of the seven metabolite compounds found in bitter melon leaves extract, two are reported to have antidepressant effects: trehalose and oleamide. Behavioral changes in antidepressant trials show that trehalose increases autophagy, a process involved in the therapeutic action of antidepressant drugs and mood stabilizers [27]. Oleamide has been reported to influence several receptors and neurotransmitter systems, particularly those operating centrally, including dopamine, serotonin, acetylcholine, gamma-aminobutyric acid (GABA), cannabinoids, and vanilloids. In a forced swim test, acute triple intraperitoneal delivery of Oleamide (10 mg/kg) resulted in a substantial decrease in the duration of immobility in mice, suggesting an antidepressant effect [28]. Oleamide has demonstrated the capacity to enhance the amplitude of currents mediated by 5-HT_{2c}, 5-HT_{2a}, and GABA_A receptors. Furthermore, Oleamide can promote sleep, partially mediated by cannabinergic pathways [29].

Bitter melon leaves extract increases feeding time in depression model mice

In this study, the CUMS model was used to induce depression in mice. The mice were chronically exposed to unpredictable stress, which caused the mice to experience behavioral changes similar to depression. The CUMS model is the most frequently utilized approach for inducing depression. CUMS is regarded as one of the premier models for simulating human depressed states in animals, utilizing a naturalistic method to subject them to unpredictable stressors that disturb homeostasis and induce somatic, physiological, neurological, and biochemical illnesses and behaviors [15,16,30]. The Novel Suppressed Feeding (NSF) test is an effective behavioural paradigm for inducing depression-like behaviour in animal models after fasting. The evaluation analyzes the conflict between consumption and apprehension. The NSF can be utilized to investigate chronic and sub-chronic antidepressant treatments in rodent models [25,26,30]. Test results using NSF showed that administration of fluoxetine (2.5 mg/kg), extract from bitter melon leaves with doses of 200 and 400 mg/kgBW were able to increase

feeding time significantly in mice with depression models, respectively amounted to 63.10 ± 1.24 seconds (p < 0.0001), 41.70 ± 2.19 (p < 0.0001), 67, 60 ± 3.73 (p < 0.0001) compared with the vehicle group (Na-CMC 1%) whose feeding time was 18.90 ± 1.64 seconds. The NSF test results are shown in figure 2.

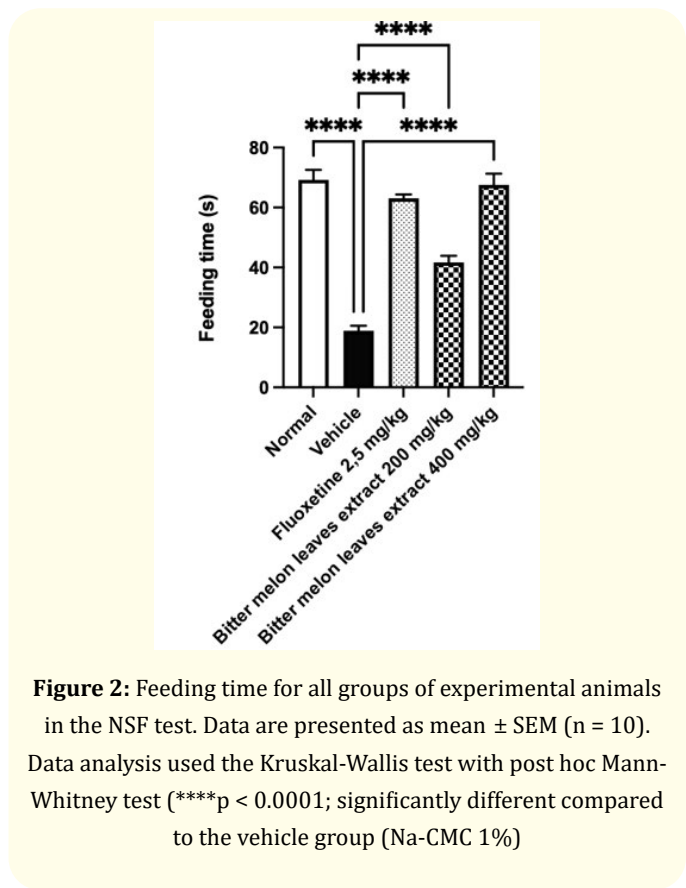


Figure 2: Feeding time for all groups of experimental animals in the NSF test. Data are presented as mean ± SEM (n = 10). Data analysis used the Kruskal-Wallis test with post hoc Mann-Whitney test (****p < 0.0001; significantly different compared to the vehicle group (Na-CMC 1%))

The feeding time observed in the group administered the 400 mg/kg dose of bitter melon leaves extract did not exhibit a statistically significant difference (p > 0.05) compared to the fluoxetine group receiving 2.5 mg/kg. In contrast to the 200 mg/kg dose of bitter melon, leaves extract group, a notable distinction was ob-

served with the fluoxetine group ($p < 0.001$); this indicates that the 400 mg/kg dose of bitter melon leaves extract demonstrates an antidepressant efficacy that is comparable to that of the fluoxetine group. To the author’s knowledge, this research is the first study to study the effect of bitter melon leaves extract on animal models of depression using the NSF test because tests that have been carried out previously by other researchers were to study the effect of bitter melon fruit on animal models of depression using the FST test by looking at the immobility time profile [31]. The results of testing the activity of bitter melon leaves in this study are in line with previous research. This research shows that bitter melon leaves have antidepressant activity by increasing eating time, while previous research shows that bitter melon fruit has antidepressant activity by reducing immobility time [31].

Bitter melon leaves extract increases BDNF levels in the hippocampus of depression model mice

The results of measuring BDNF levels are shown in figure 3; administration of fluoxetine (2.5 mg/kg) and bitter melon leaves extract 200 and 400 mg/kg was able to increase BDNF levels significantly in depression model mice, respectively by 184.59 ± 5.16 pg/ml ($p = 0.0068$), 193.14 ± 10.61 pg/ml ($p = 0.0014$), 202.75 ± 8.04 pg/ml ($p = 0.0002$) compared to the vehicle group (Na-CMC 1%) whose BDNF levels were 145.24 ± 8.42 pg/ml. BDNF levels in the fluoxetine (2.5 mg/kg), bitter melon leaves extract 200, 400 mg/kg groups showed no significant differences ($p > 0.05$) with the normal group $199, 73 \pm 2.80$ pg/ml. BDNF levels in the bitter melon leaves extract (200, 400 mg/kg) showed no significant difference ($p > 0.05$) with the fluoxetine group (2.5 mg/kg). This research shows that bitter melon leaves extract at doses of 200 and 400 mg/kg has effectiveness as an antidepressant comparable to the drug fluoxetine (2.5 mg/kg) by increasing BDNF levels in the hippocampus. Unpredictable chronic stress can decrease BDNF expression [22]. Many studies have demonstrated that BDNF, which is found in the nervous system, functions as an intercellular messenger responsive to stress and is a key component of the stress response response. It was shown that persistent antidepressant therapy was associated with a considerable rise in the levels of BDNF mRNA in the dentate gyrus of the dorsal hippocampus. This finding highlights the function that BDNF plays in the mechanisms that underlie the activity of medication for depression. When antidepressant medication is administered, the effects of stress are reversed or blocked. Stress causes decreased in production of BDNF in limbic areas that con-

trol disposition. The atrophy of certain limbic tissues, particularly the hippocampus, may be a contributing factor in the development of depression in individuals who have decreased levels of BDNF. Previous studies have shown that animals who were given CUMS as a treatment showed a reduction in BDNF levels. This research is consistent with those findings. At the same time, the antidepressant medication fluoxetine and the extract of bitter melon leaves showed the ability to raise levels of BDNF in the hippocampus of test mice that were given CUMS [32-34]. Other studies also show increased BDNF in the hippocampus has a behavioral change effect. BDNF is a protector for vulnerable and damaged hippocampal neurons during depression. In addition, BDNF can change the release of neurotransmitters that can activate postsynaptic neurons so that it has potential protective functional consequences on the hippocampal circuit. BDNF contributes to memory and cognition related to depression; thus, elevated BDNF levels correlate with neurogenesis, cellular survival, and dendritic branching [35]. This work demonstrates that the injection of bitter melon leaf extract at doses of 200 and 400 mg/kg can elevate BDNF levels in the hippocampus and produce behavioural alterations in mice exposed to chronic unpredictable mild stress (CUMS), so with these findings, bitter melon leaves can be used as candidates for antidepressant drugs.

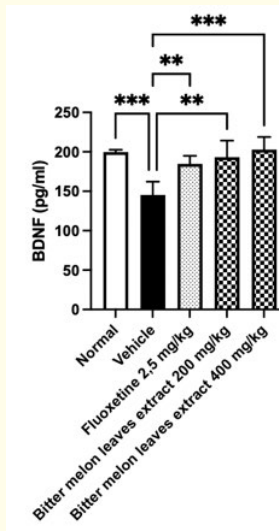


Figure 3: BDNF levels in the hippocampus of all groups of experimental animals. Data are presented as mean SEM ($n = 4$). Data analysis used oneway-ANOVA test with post hoc Tukey test ($***p < 0.001$; $**p < 0.01$) was significantly different compared to the vehicle group (Na-CMC 1%).

Conclusion

This study's results indicate that bitter melon leaves extract contains several active compounds and has antidepressant activity, as demonstrated by an increase in feeding time in the mouse model with depression and an increase in BDNF levels after bitter melon leaves extract treatment. This shows that bitter melon leaves extract contains active compounds that have the potential to be developed as antidepressant drug candidates.

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Conflict of interest

The authors have declared that there are no competing interests.

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