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ANTIDEPRESSANT ACTIVITY OF 80% BITTER MELON-LEAF ETHANOL EXTRACT (MOMORDICA CHARANTIA L.) AND B -SITOSTEROL COMPOUND IN VIVO

Ani Kristiyani[1,2] , Zullies Ikawati[1*], Andayana Puspitasari Gani[3] , Zaenal Muttaqien Sofro[4]
[1] Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada
[2] Faculty of Pharmacy, Universitas Kristen Immanuel, Yogyakarta
[3] Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada
[4] Physiology Department, Faculty of Medicine, Nursing, and Public Health, Gadjah Mada University,
zullies_ikawati@ugm.ac.id

Abstract
Depression is a psychiatric disorder that affects mood and physical health and contributes greatly to the global burden of disease. Herbal remedies can be used as an alternative to the treatment of depression. The purpose of this study is to determine the activity of 80% ethanol extract of bitter melon leaves (*Momordica charantia* Linn) as an antidepressant and β-sitosterol compound which is a marker compound in bitter melon leaf extract. In this study, β-sitosterol compounds in bitter melon leaf extract were identified qualitatively using Fourier Transform InfraRed (FTIR) and in vivo antidepressant activity tests. The in-vivo test used 30 mice with a weight of 20-30 grams which were randomly divided into 6 groups, namely the normal group (not receiving treatment), the negative control group (Na-CMC 1%), the positive control group (fluoxetine 2.5 mg/kg), the β-sitosterol group (β-sitosterol 30 mg/kg), the bitter melon leaf extract group dose 1 (200 mg/kg), and the group of bitter melon leaf extract dose 2 (400 mg/kg). The mice received stressors using the Chronic Unpredictable Mild Stress (CUMS) method for 28 days. On the 15th day after the mice received CUMS, the mice were exposed to test extracts and drugs for 14 days according to the treatment group. Weight measurements were taken on day 1 and on day 29. The behavioral test used the Forced Swimming Test (FST) and Novel Suppressed Feeding (NSF) with feeding time parameters, and the immobility time was carried out on the 29th day, in addition to that the number of necrosis in gastric and the number of hippocampal pyramidal cells were also measured. The results showed that at all absorption peaks in the FTIR of bitter melon leaf extract, there were -OH, C-H, C=C, - CH2-, C-O groups which are the characteristic peaks of the β-sitosterol compound. In weight measurement, bitter melon leaf extract had antidepressant activity by not causing significant weight changes compared to the normal group ($P>0.05$). The β-sitosterol and bitter melon leaf extract group dose 2 had antidepressant activity by maintaining appetite which was shown with a higher feeding time value compared to the negative group ($P<0.05$). The positive control group, β-sitosterol, and bitter melon leaf extract doses 1, and 2 when compared to the negative control group, although not significant ($P>0.05$) had antidepressant activity values by lowering despair indicated by smaller immobility time values, lowering the number of necrosis in gastric lower and protecting the hippocampal pyramidal cell count higher. Conclusion: 80% ethanol extract of bitter melon leaves contains active compounds β-sitosterol and has activity as an antidepressant by not causing weight changes, maintaining appetite, lowering despair, protecting gastric from necrosis and protecting hippocampal pyramidal cells.

Keywords**:** *depresi, behavioral, β-sitosterol, CUMS, FST, NSF, Momordica charantia Linn*

* Corresponding author :
Address : Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara, Yogyakarta 55281, Indonesia
Email zullies_ikawati@ugm.ac.id

INTRODUCTION

Depression is a psychiatric disorder with an estimated lifetime prevalence of 10% in the general population (Kessing, 2007). Symptoms of depression consist of both somatic and non-somatic factors. Somatic factors include difficulty sleeping, changes in appetite, weight, poor concentration, fatigue, and (Berken et al., 1984) psychomotor agitation/retardation. Non-somatic factors consist of affective items such as depressive mood, anhedonia, feelings of worthlessness, and thoughts about death (Elhai et al., 2012). It is estimated that 3.8% of the world's population or about 280 million people are depressed, and more than 700,000 people are experiencing suicide. Suicide is the fourth leading cause of death in 15-29 years old (WHO, 2023). Based on the Sample Registration System conducted by the Health Research and Development Agency in 2016, suicide data was obtained per year of 1,800 people or every day 5 people committed suicide, and 47.7% of suicide victims were at the age of 10-39 years which is the age of adolescents and productive age (Kemekes, 2021).

Weight changes, including weight loss and gain are one of the parameters observed in depressive symptoms. Weight changes in depressed patients are caused by dietary changes, appetite disorders, nutritional disorders, and behavioral changes (Felton et al., 2010). Analysis of cDNA microarrays revealed that chronic stress of restraint affects the expression of genes in the hypothalamus associated with weight control (Jeong et al., 2013). Depression causes behavioral and dietary changes that can lead to gastric disorders (Ruan et al., 2023). Changes in behavior, lifestyle, and eating disorders are related to necrosis in the stomach (Lee et al., 2011); (Amori, 2007). Necrosis is the death of cells due to irreparable exposure to stimuli (Adigun et al., 2023). The hippocampus is an important part that is often noticed in research on depressive disorders, where hippocampus pyramidal cells are susceptible to exposure to conical stress that results in depression (Arjadi et al., 2014). Hippocampus cells are pyramidal in shape located in each amminis cornu (Standring, 2005).

The specific goals of each phase of depression treatment are acute, advanced, maintenance, as a strategy map for managing depressed patients. In mood disorders, the goals of initial treatment include symptoms of remission (acute phase), restoration of psychosocial function (acute and advanced phase), prevention of relapse (advanced phase) or new episodes in patients with recurrent depression (maintenance phase) (Rush, 2004). With proper treatment, 70-80% of individuals with depressive disorders can significantly reduce symptoms. Fluoxetine is an antidepressant drug of the Selective Serotonin Reuptake Inhibitors (SSRIs) class which is a first-line agent for the treatment of depression (Sangkuhl et al., 2009). Fluoxetine is generally well tolerated but has the most common side effects being dry mouth, nausea, and weight gain (Zou et al., 2013); (Gramaglia et al., 2018).

Bitter melon leaf (*M. charantia* Linn.) is reported to have antidepressant effects that depend on the serotonergic system (5-HT2 receptors), noradrenergic (α1- and 2-adrenoceptors), dopaminergic (D2 receptors), and muscarinic cholinergic systems, and benzodiazepine-like anxiolytic effects (Ishola et al., 2014a). The antidepressant effect of methanol extract of bitter melon leaf (*M. charantia* Linn.) at a dose of 300 mg/kg was comparable to imipramine at a dose of 5 mg/kg in Swiss albino male rats (Ganesan et al., 2007). Charantin isolated from *M charantia* Linn was shown to have a neuroprotective effect (Tamilanban et al., 2018). Charantin consists of glucosides β-sitosterol and 5,25-stigmasteryl glucoside in equal amounts (Tamilanban et al., 2018). β-sitosterol has been reported as an active compound that has antidepressant effects (Yin et al., 2018); (Zhao et al., 2016).

Previous studies have shown the activity of bitter melon leaves (*M. charantia* Linn.) against depressive disorders but is still limited. Based on the currently available evidence, it is important to conduct research to find out and study the effects of bitter melon leaves as an antidepressant agent. This study aims to determine whether there is a content of β-sitosterol compounds in 80% ethanol extract of bitter melon leaves (*M. charantia* Linn) and to determine the effectiveness of bitter melon leaf extract (*M. charantia* Linn) on depression in a mouse model induced by stress using CUMS

METHOD

This research was conducted at the Laboratory of Histology and Cell Biology, Laboratory of Animal Experiments, Faculty of Medicine, Public Health and Nursing, Gadjah Mada University. The research has received ethics committee approval with Ref. No: KE/FK/IG11/EC/2023. This research method uses an experimental method with research stages, namely: making 80% bitter melon leaf ethanol extract, qualitatively identifying β-sitosterol compounds in bitter melon leaf extract, testing antidepressant activity in vivo through observation of weight changes, behavioral with the NSF

method, FST then followed by total tracing of gastric necrosis and hippocampal pyramidal cells. The data obtained was statistically analyzed with the Statistical Product and Service Solution (SPSS) program.

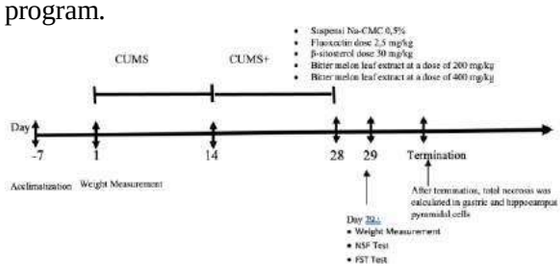


Figure 1. Scheme of in vivo testing

Material

Simplisia bitter melon leaves were purchased at BALITRO Bogor, West Java, which was determined at the Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, 80% ethanol for the manufacture of extracts. The in-vivo test used male mice (Mus musculus) with the Balb/c strain, 4-5 weeks old with a body weight of 20-30 kg ± 10% grams, with a total of 30 mice and divided into 6 groups. In the maintenance process, bedding in the form of rice husks, feed in the form of pellets (RatBio®) and mineral water (Aqua®) are needed. The suspension and test solution were made using fluoxectin (OGB Dexa®), β-sitosterol, ethanol extract 80% bitter melon leaf and Na CMC 1% (Sigma-Aldrich®, USA), Materials used for CUMS depression induction include: feed in the form of pellets (RatBio®) and mineral water (Aqua®), sawdust, clean water, water at a temperature of 31 °C. Materials used for behavioral tests include tissue, water, pellets. The materials used to measure total necrosis, and pyramidal cells are gastric organs, brain organs, NaCl, PBS, and formaldehyde.

Tool

The tools used for the preparation of extracts include stirring rods, analytical balances as well as glassware, Batch Sonicator, thermometer. Identify β-sitosterol compounds using the FTIR (Thermo Scientific Nicolet iS10) tool. CUMS induction using equipment includes stopwatches, mouse cages, tail clamps, aquariums, cage casing boxes, plasters. Weight measurement using a digital scale (Sf-400). Behavioral testing uses NSF test tools and FST test tools. The NSF test apparatus is made of acrylic square open box (100 × 10 × 10 cm, length × width × height) consisting of a dining area, a drinking area, a recording camera, which is attached to the device and is attached to the Miconos® application, stopwatch and a computer set. The FST test equipment consists of an aquarium with dimensions of 40x25x25 cm. Measurement of total gastric necrosis and total hippocampal pyramidal cells using microskup (Olympus CX23).

Research procedure:

1. Preparation of bitter melon leaf extract
   The preparation of bitter melon leaf extract uses the Ultrasonic Assisted Extraction method which is a modified maceration method by adding ultrasound (a signal with a high frequency of 20 kHz).
2. Qualitative test of β-sitosterol compounds in bitter melon leaf extract
   Qualitative test of bitter melon leaf extract using an FTIR tool with FTIR scanning stages with a wave number of 4000-400 cm-1, resolution 8, scan 32, final format absorbance, sample preparation, and sample test
3. Modeling depressive conditions
   Modeling of depressive conditions in mice using the CUMS method. The mice were exposed to different stresses for 28 days according to a random schedule to avoid any kind of habituation. The mice are left undisturbed in their cages except for treatment tests. Stress exposure is as shown in table 1 below: (Qu et al., 2021) (Wu et al., 2012) (Gao et al., 2021) (Zhang et al., 2021)

Table 1. Stress Exposure Plan

Stressor	Information
1	Food shortages (24 hours)
2	The cage with sawdust is soaked with 125 ml of clean water (24 hours)
3	Cage without sawdust (24 hours)
4	Swim in warm water at 30°C
5	Emptying a bottle (4 hours)
6	Reduced drinking water (24 hours)
7	30° tilted cage without base (24 hours)

4. Weight measurement
   Weight measurement was done on days 1 and 29
5. Administration of test materials and drugs
   On the 15th day after the mice received CUMS, the mice were exposed to test extracts and drugs for 14 days according to the treatment group.
6. Behavioral
   1) NSF
   The mice were fasted and acclimatized for 24 hours in the testing room. Each mouse was placed in the test device in the same position and direction. The feeding time or the time it takes for each mouse to eat the pellet is recorded for 5 minutes, in this test the behavior of not eating (e.g. touching and kissing) is not considered eating (Qu et al., 2021)
   2) FST
   Each mouse was placed in the test device, the number of immobility episodes was recorded and evaluated based on the mice's swimming behavior for 5 minutes in the test device (Sasaki et al., 2021)
7. Total measurement of gastric necrosis and hippocampal pyramidal cells
   The number of gastric necrosis and hippocampus pyramidal cells was calculated

using a microscope with a magnification of 400x, as much as 1 field field for each sample.

RESULT AND DISCUSSION

Qualitative analysis of bitter melon leaf extract

FTIR is an instrument that can be used to find the functional group of the β-sitosterol compound. Medicinal plants consist of various chemical compounds, some of which can absorb energy from IR rays at a certain number of wavelengths. The FTIR spectrum has a wavelength number of 4000-400 cm-1 (Rodriguez-Saona et al., 2016) . The structure of β-sitosterol is shown in Figure 2, while the FTIR characteristic spectrum of bitter melon leaf extract is shown in Figure 3 below:

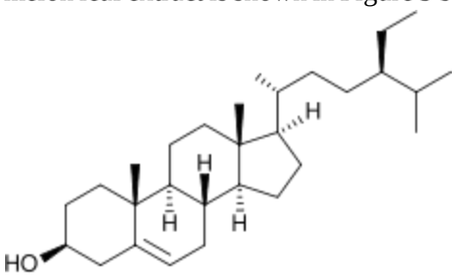


Figure 2. Chemical structure β-sitosterol

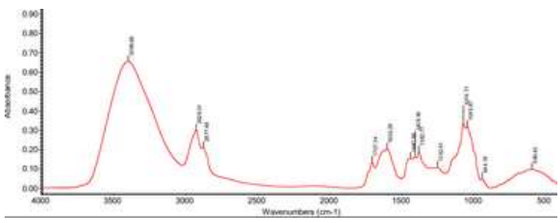


Figure 3. FTIR spectrum of bitter melon leaf extract

The FTIR results of bitter melon leaf extract showed absorption with wavelengths at different numbers. The peak absorption of the FTIR spectrum of bitter melon leaf extract is matched with the theoretical wave number along with the approximate functional group possessed by the β-sitosterol (Coates, 2000) compound, summarized in table 2 below:

Table 2. Peak spectrum of FTIR bitter melon leaf extract, theoretical wave number, approximate functional group, and indication of β-sitosterol compound.

No.	Number of waves (cm^{-1})	Number of theoretical waves (cm^{-1})	Approximate functional clusters	Compound indications
1	3398,68	3400-3200	-OH H-bonded	β-sitosterol
2	2928,01	3000-2850	C-H alkena (stretch)	β-sitosterol
3	2877,49	2900-2800	C-H alkana (stretch)	β-sitosterol
4	1707,34	1680-1600	C=C alkena	β-sitosterol
5	1603,29	1680-1600	C=C alkena	β-sitosterol
6	1440,89	1465	-CH2- (bend)	β-sitosterol
7	1409,36	1450 and 1375	C-H di -CH3 (bend)	β-sitosterol
8	1382,77	1450 and 1375	C-H di -CH3 (bend)	β-sitosterol
9	1252,61	1300-1000	C-O alcohol	β-sitosterol
11	1045,87	1300-1000	C-O alcohol	β-sitosterol
12	944,18	1000-650	C-H alkena (out of plane bend)	β-sitosterol
13	599,42	1000-650	C-H alkena (out of plane bend)	β-sitosterol

In the FTIR results of bitter melon leaf extract, several absorption peaks were found which are estimated to be functional groups that correspond to the characteristics of the β-sitosterol compound. These findings are supported by previous research in which β-sitosterol is an active compound found in bitter melon plants in large quantities; (Yoshime et al., 2019) (Yoshime et al., 2016)

Weight changes

Table 3. Weight changes on day 1 and day 29

Group	Average Weight Day 1 n = 5	Average Weight Day 29 n = 5	Average Weight Change n = 5	Elementary Average Weight Change n = 5
Normal	35,50	45,00	9,50	4,04
Na-CMC 1%	34,60	42,40	7,80	5,71
Fluoxetine (2,5 mg/kg)	34,00	45,00	11,00	6,30
β-sitosterol (30 mg/kg)	33,00	42,20	9,20	5,55
Bitter Melons Leaf Extract dose 1 (200 mg/kg)	33,20	37,80	4,60	2,60
Bitter Melons Leaf Extract dose 2 (400 mg/kg)	31,00	33,60	2,60	5,54

one way ANOVA (P<0,05)

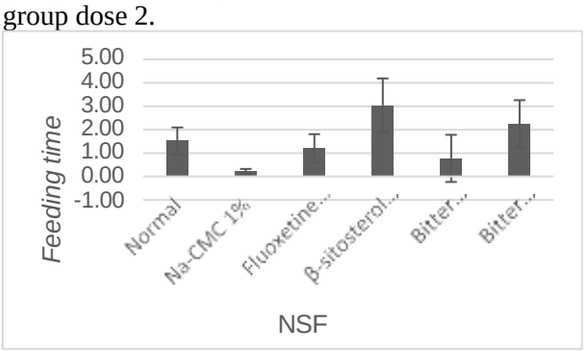
Weight changes related to depressive symptoms, including weight loss and gain are one of the parameters observed in depressive symptoms (Felton et al., 2010) . The research conducted by Zhu et al, showed that the depression modeling method affects weight loss that occurs at different times. The mice that received CUMS experienced significant weight loss compared to normal mice at week 5 (P<0.01). In this study, the measurement of weight change was carried out on the 29th day (week 4), the positive group, β-sitosterol, bitter melon leaf extract dose 1 and dose 2 did not experience significant weight changes when compared to the normal group (P>0.05), thus showing antidepressant activity by maintaining weight. The 1st and 2nd dose of bitter melon leaf extract group, although not significant (P>0.05) weight gain was lower than that of the other group, (Zhu et al., 2019) this is because bitter melon leaf extract has activity against weight loss by mechanism through inhibition of fat synthesis, increased glucose utilization and stimulation of lipid-lowering activity, improvement of insulin resistance, fat accumulation, inflammation of

adipose tissue; ; . Research (Fan et al., 2019) (Bao et al., 2013) (Bao et al., 2013) on herbs for depression conducted by Liao et al showed that herbs that have activity as antidepressants do not lose weight in test animals (Liao et al., 2023) , some other studies have actually shown that antidepressant drugs given to depressed patients have side effects on weight gain and metabolic syndrome. In contrast to the research of Liao et al; Berken et al, and Gramaglia et al, Bitter melon leaf extract showed activity against depression and activity against weight loss; , this suggests that bitter melon leaf extract may be an option as an antidepressant drug candidate in patients at risk of weight gain and metabolic syndrome problems. (Berken et al., 1984); (Gramaglia et al., 2018) (Ishola et al., 2014b) (Fan et al., 2019)

Behavioral changes

1. Novelty suppressed feeding (NSF)

Feeding time is a parameter observed in the test using the NSF method. *Feeding time* is the length of time the mice eat at the time the test takes place, where the low desire to eat is a form of behavior of the mice who are depressed. Decreased *feeding time* is a form of stress and can lead to a decrease in phosphorus-mTOR in the prefrontal cortex, a molecular signal associated with acute depression; Antidepressant drugs are expected to prolong (Ren et al., 2022) (Shin et al., 2022) *the feeding time*. In this study, the group of mice that had the highest time to eat was in the β-sitosterol group, followed by the bitter melon leaf extract group dose 2.



one way ANOVA (p<0,05)

Figure 4. Feeding time in NSF test

In the NSF test, the feeding time measurement was carried out for 5 minutes, before the NSF test was carried out, the mice were fasted for 24 hours, so that the results of this feeding time were not only due to depression but also influenced by the desire to maintain appetite after the mice were fasted, where mice that were depressed had a low appetite. The negative control group compared to the other group had the least feeding time value, which showed that the negative control group had greater depressive symptoms than the other group. The ANOVA one-way statistical test showed that there was a significant difference between the groups (P<0.05), then the post hoc test showed a significant difference (P<0.05), namely between the negative group and the β-sitosterol group and bitter melon leaf extract dose 2 (400 mg/kg). This suggests that β-sitosterol and bitter melon leaf extract dose 2 have the potential to maintain appetite, which can be linked to activity as an antidepressant. The bitter melon leaf extract group dose 2 has a fairly high feeding time value but the weight measurement shows the smallest weight value, this is because bitter melon leaf extract in addition to having the activity of maintaining appetite also has weight loss activities; , so that bitter melon leaf extract works as an antidepressant through the potential to maintain appetite but not increase weight. Other studies show that some herbs have activity against depression with comparable and even better risk profiles, benefits than fluoxetine (Bao et al., 2013) (Fan et al., 2019) (Sarris et al., 2011); (Setorki, 2020).

2. Forced Swimming Test (FST)

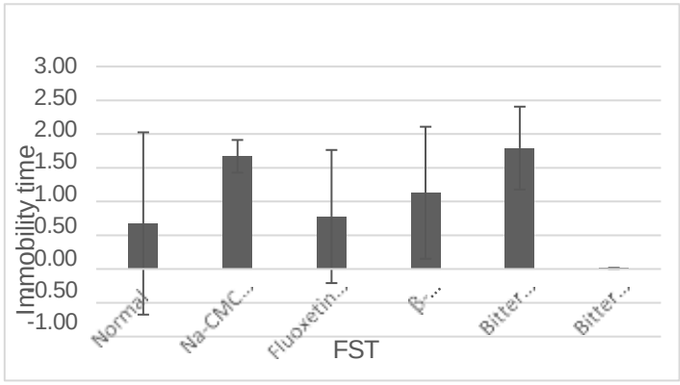


Figure 5. Immobility time on FST test

In testing the effectiveness of an antidepressant drug, the FST test method is often used. The parameter observed in the FST test is *immobility time* which is a form of despair due to exposure to stress that causes severe depression. The administration of antidepressant drugs is able to have an effect on decreasing (Yankelevitch-Yahav et al., 2015) *immobility time* and increasing movement time (Rodrigues et al., 2023). The results of the FST test showed that the positive control group, β-sitosterol, and bitter melon leaf extract dose 2 (400 mg/kg) had *a smaller immobility time* compared to the negative control group, although not significant (P>0.05), this suggests that the positive control group, β-sitosterol, bitter melon leaf extract dose 2 (400 mg/kg) had the potential to reduce the existing breakage in the face of stress exposure associated with antidepressant activity. The FST test method has the advantage that it is relatively easy to do and the results can be analyzed quickly, while the disadvantages of this method include chronic depression problems that have an impact on brain structure/function, so to see the effectiveness of an antidepressant drug requires an examination of brain structure/function (Yankelevitch-Yahav et al., 2015)

Necrosis in the stomach and pyramidal cells in the hippocampus

Necrosis is the death of cells due to irreparable exposure to stimuli. Depression is not only caused by changes in neurotransmitter levels but also has a role in cell death (Adigun et al., 2023) (Hidayat et al., 2020) . An increase in the number of cells in the stomach that undergo necrosis is related to changes in behavior and food intake which are symptoms of depression; Based on the results of histopathological examination of the stomach of mice, gastric necrosis were found in all groups (figure 7). Based on (Lee et al., 2011) (Loves, 2007) *the Kruskal Wallis* statistical test , there was no significant difference between groups (P>0.05), but the number of gastric necrosis showed that the normal, positive control, β-sitosterol, bitter melon leaf extract doses 1 and 2 had lower necrosis compared to the negative control group. This suggests that the positive control group, β-sitosterol, bitter melon leaf extract doses 1 and 2 (400 mg/kg) have potential protection against cell death.

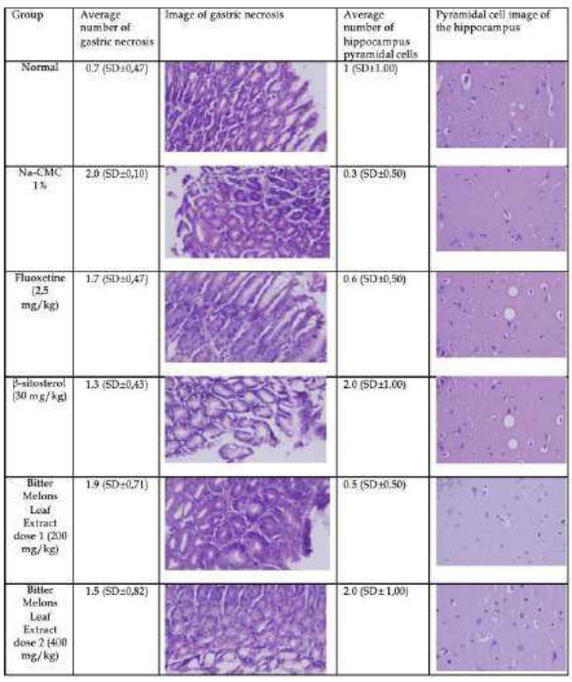


Figure 7. Histology of the number and image of necrosis in the stomach and pyramidal cells in the hippocampus

The hippocampus is an important part of the research of depressive disorders, the pyramidal cells of the hippocampus are susceptible to exposure to conical stress that results in depression (Arjadi et al., 2014). Exposure to stressors can decrease the number of pyramidal cells in the hippocampus (Arjadi et al., 2014). Based on the Kruskal Wallis statistical test, there was no significant difference between the groups (P>0.05), but the number of hippocampus pyramidal cells showed that the normal, positive control, β-sitosterol, bitter melon leaf extract doses 1 and 2 had higher necrosis cells than the negative control group. This suggests that the positive control group, β-sitosterol, bitter melon leaf extract doses 1 and 2 has potential protection against hippocampal pyramidal cell susceptibility due to stress exposure that causes depression. Another study reported that hippocampus pyramidal cells are in each region of the ammonis cornus (CA) including CA1, CA2, and CA3, each with a different level of susceptibility. Pyramidal cells in the CA1 and CA2 regions are susceptible to hypoxia, while pyramidal cells in the CA3 region are susceptible to physical stressors and exposure to chronic stress in the hippocampus resulting in depression and loss of neurons in the hippocampus and amygdala, thereby decreasing memory and cognitive function. This study was not counted separately on each pyramidal cell in the CA1, CA2, and CA3 regions, but was calculated as a whole, so the total results of the pyramidal cells obtained can be affected by various vulnerabilities and cannot describe the specific number of pyramidal cells exposed to CUMS stress (Suparno, 2008).

CONCLUSION

All absorption peaks in FTIR of bitter melon leaf extract were found to have -OH, C-H, C=C, -CH2-, C-O groups which are characteristic peaks of the β-sitosterol compound. In weight measurement, bitter melon leaf extract did not experience significant weight changes compared to the normal group (P>0.05), this shows that bitter melon leaf extract has the potential as an antidepressant because it does not cause significant weight changes. The β-sitosterol group and bitter melon leaf extract dose 2 had a higher feeding time value than the negative group (P<0.05), which showed that β-sitosterol and bitter melon leaf extract dose 2 had the potential to maintain appetite, which could be related to activity as an antidepressant. The positive control group, β-sitosterol, and bitter melon leaf extract doses 1, and 2 compared to the negative group although not significant (P>0.05) had antidepressant activity values by lowering desperate indicated by smaller immobility time values, lowering the amount of necrosis in gastric lower and protecting the hippocampal pyramidal cell count higher. The results of this study concluded that 80% ethanol extract of bitter melon leaves contains active compounds β-sitosterol and has activity as an antidepressant by not causing weight changes, maintaining appetite, lowering despair, protecting gastric from necrosis and protecting hippocampal pyramidal cells.

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