Trad. Med. J., January-April 2021
Vol. 26(1), p 13-20
ISSN-p : 1410-5918 ISSN-e : 2406-9086

Submitted : 20-12-2019
Revised : 04-03-2021
Accepted : 24-03-2021

Radical Scavenging Activity and Acute Toxicity of Bitter Melon (*Momordica Charantia* L.) Seeds Oil

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ABSTRACT

Bitter Gourd or bitter melon (Momordica Charantia L.) is a common type of vegetable which is safe for daily consumption. The seeds are bitter gourd part that useless. Research on bitter melon seeds oil has promising commercial applications. This study aims to determine the radical scavenging activity and safety of bitter melon seeds oil through acute toxicity study. The content of phenols, flavonoids, and antioxidant activity was analyzed. The radical scavenging activity was evaluated using the DPPH method. Antioxidant activity is expressed as an IC-50 value. The results showed that the phenol content of bitter melon seeds oil was 0.0118 ±0.0006%, the flavonoid content was 0.0127±0.0004%. From the radical scavenging activity study, the IC-50 of bitter melon seeds oil was 11.31 ± 0.77 mg/ml. The results of this radical scavenging activity study showed very weak activity. The results of the acute-toxicity study show the LD-50 cannot be determined precisely because up to the highest dose of 100 ml/kg does not cause death even though it causes toxic symptoms such as diarrhea. Overall, the results indicate that bitter melon seeds oil is a compound that is categorized as practically non-toxic with low antioxidant activity.
Keywords: bitter melon sedds oil; total phenol content; flavonoid content; radical scavenging activity; acute toxicity

INTRODUCTION

Herbal plants have been used for a long time as a traditional medicine to treat various diseases. Bitter melon is a vegetable widely used in Africa and Asia as a plant for medicine (Beloin *et al*., 2005). One of the common uses of bitter melon in medicine is as an antidiabetic (Khan *et al*., 2019). Hypoglycemic activity in vivo was reported for the fruit, seeds, and leaves (Xiang *et al*., 2007). Bitter melon seeds are parts that are often discarded and not utilized. In contrast, bitter melon seeds contain edible oil which has a variety of biomedical benefits, such as proven in vitro to have antidiabetic activity (Ahmad *et al*., 2012) and can reduce blood lipids and liver (Noguchi *et al*., 2001).

Studies show that bitter melon seeds are rich in conjugated α-linolenic acid, polyphenolic such as gallic acid, gentisic acid, catechin, and epicatechin, apigenin, luteolin (Horax *et al*., 2005), hence it is widely used in ancient medicine for the treatment of many diseases such as diabetes and atherosclerosis (Bialek *et al*., 2014). Lipid profiles of bitter melon seeds also contain high amounts of polyunsaturated fatty acids (PUFA) (Jing *et al*., 2011) and other bioactive compounds such as tocopherol and polyphenols (Anjum *et al*., 2013). Polyphenols and tocopherols are compounds that have antioxidant activity. This antioxidant ability is very beneficial because used to treat various degenerative diseases such as diabetes mellitus, atherosclerosis, and cancer (Lee *et al*., 2016). Polyphenol compounds find in the seeds. The presence of various polyphenol compounds in bitter melon seeds allows these polyphenol compounds to also present in bitter melon seeds oil. Therefore, the total polyphenol content, total flavonoids, and free radical scavenging activity test using the DPPH method were carried out on bitter melon seeds oil.

Antioxidant activity was reported in a bitter melon water extract. The total phenol content in water extract was 13.28 GAE/g and had an effective inhibition of DPPH free radical. High phenols, flavonoids, and activity against DPPH free radicals were found in mature bitter melon fruit. The phenolics and flavonoids content proves that bitter melon is a source of natural antioxidants that can provide potential health benefits (Lee *et al*., 2016).

Although herbal products are known to be safer, all ingredients, whether it is synthetic or natural products, will be toxic if not given proportionally. A toxicity study to determine the adverse biological effects resulting from the administration of a substance in a short time must be established. In this study, an acute toxicity test is conducted to determine the Lethal Dose (LD50).

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Therefore the aim of this study is to evaluate the antioxidant potential of bitter melon seeds oil in vitro and determine the limit of toxicity (LD50) of bitter melon seeds oil to support its development as a nutraceutical and herbal medicine.

METHODOLOGY

Materials

Bitter melon seeds oil obtained from PT. Nature in Bottle India, Female mice Balb/c strain with the weight of approximately 20 g obtained from the Biomedical Laboratory of the Faculty of Pharmacy, University of Jember. Gallic acid was purchased from Sigma (St. Louis, MO, USA), sodium hydrogen carbonate from BDH Supplies Laboratory, UK, while potassium acetate, aluminum chloride, Folin Ciocalteu-reagent, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma (St. Louis, MO, USA).

Methods

Bitter melon seeds oil phytochemical screening

The phytochemical screening of bitter melon seeds oil aims to determine the content of secondary metabolites. The screening was carried out with color reagents to determine the presence or absence of alkaloids, terpenoids, and flavonoids. The phytochemical screening method was carried out according to Harbone (1987).

Determination of Total Phenolic Content

The calibration curve was prepared from Gallic acid working standards of 20.0, 40.0, 60.0, 80.0, and 100.0 ppm. One gram of bitter melon oil was put into a tube and added with n-hexane (1:1), then mix in vortex 1-2 minutes. The mixture was extracted with 3 ml of methanol: water (4:1 v/v) and re-vortexed for 1-2 minutes. The mixture was then centrifuged at 3000 rpm for 10 minutes. The supernatant (water phase) was taken and the extraction repeated twice until a 10 mL supernatant was obtained in the flask. One ml of a test solution and a standard solution were pipetted separately, to each solution added with 0.5 ml of the folin-ciocalteu reagent. After 3 minutes, 1.5 mL of 7.5% Na2CO3 solution was added to the mixture and let stand for 2 hours. The absorbance was measured at the wavelength of 765 nm. The blank (methanol: water 4:1) measures in the same way. The total phenolic content of the sample was expressed as % w/w.

Determination of Flavonoid Content

The calibration curve was prepared from Quercetin working standards of 30.0, 40.0, 50.0, and 80.0 ppm. One gram of bitter melon oil is put into a tube and added with n-hexane (1:1). The mixture was mixed in vortex 1-2 minutes. The mixture is extracted with 3 ml of methanol: water (4: 1 v/v) and re-vortexed for 1-2 minutes. The mixture is then centrifuged at 3000 rpm for 10 minutes. The supernatant (water phase) is taken. Repeat the extraction twice until a 10 mL supernatant is obtained in the measuring flask. The test solution and standard solution were pipetted 0.5 ml separately, then added with 1.5 ml of ethanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1M sodium acetate, and 2.8 ml of distilled water. The mixture was shaken and stand for 30 minutes at room temperature. The absorbance was measured at a wavelength of 425 nm. The blank was measured in the same way without the addition of aluminum chloride. The total flavonoid content of the sample is expressed in percentage w/w.

Determination of Radical Scavenging Activity Using DPPH Method

Three grams of the bitter seeds oil is put into a tube and added with n-hexane (1:1). The mixture was mixed in vortex 1-2 minutes. The mixture is extracted with 3 ml of methanol: water (4: 1 v/v) and re-vortexed for 1-2 minutes. The mixture is centrifuged at 3000 rpm for 10 minutes. The supernatant (water phase) is taken. The extraction was repeated twice until a 5 mL supernatant is obtained in the measuring flask. The extracted solution was diluted into several concentrations. A total of 600 µL of the sample was mixed with 2.4 mL of DPPH 0.004% until homogeneous, and the absorbance was observed at a wavelength of 517 nm after 5 minutes. The free radical scavenging activity was calculated using equation 1.

$$Scavenging\ activity\ (\%) = \left(\frac{Abs_{control} - Abs_{sample}}{Abs_{control}}\right)$$

(Equation 1)

Where $Abs_{control}$ is the absorbance of the DPPH radical, Abs_{sample} is the absorbance of the DPPH radical solution mixed with the sample.

A value of 0% means it does not have free radical scavenging activity, while a value of 100% means total reduction, and testing needs to be continued with the dilution of the test solution to see the concentration limit of its activity. Subsequently, a curve was made between the concentration of the test solution and the % of DPPH reduction. The IC-50 value was determined as the concentration of the test solution that gave 50% DPPH reduction. The IC-50 value is commonly used to express the antioxidant activity of a sample

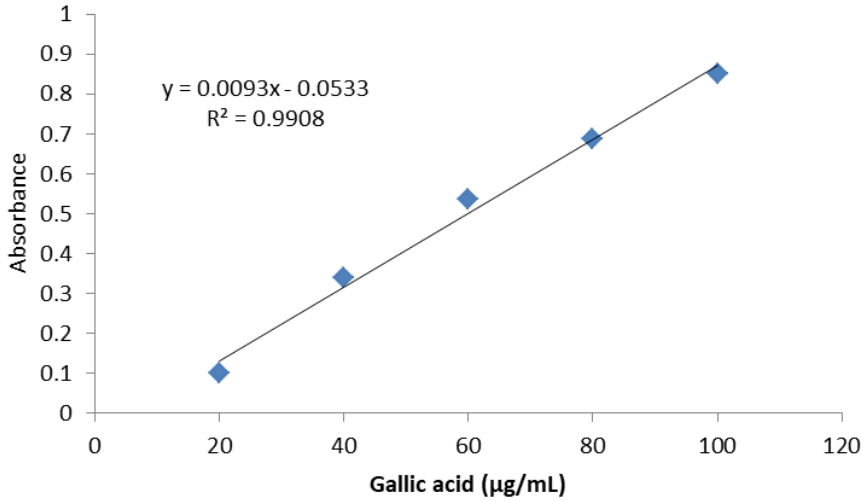


Figure 1. Total phenolic content for standard gallic acid

Table I. Results of phytochemical screening

No.	Phytochemical study	Result	Conclusion
1	Alkaloids	Formation of orange sedimen	(+)
2	Triterpenoids	Formation of brown ring	(+)
3	Flavonoids	Formation of a red or orange color on the amyl alcohol layer	(+)

by the DPPH free radical reduction (Molyneux, 2004).

Acute Toxicity Study

The principle of acute toxicity study is the administration of a sample with various dosage levels once during the test period to several groups of animals. The next step is to observe the toxic effects and death. Animals that died during testing and lived until the end of the testing period were sacrificed for evaluation. Animals were divided into 5 groups consisting of control groups and groups that were given oral bitter seeds oil with various dose levels. Group 1. Control group; Group 2. A single oral dose of 12.5 ml/Kg; Group 3. A single oral dose of 25 ml/Kg; Group 4. A single oral dose of 50 ml/kg; Group 5. A single oral dose of 100 ml/kg.

Observation of the toxic effects of drug administration is carried out during the first 3 hours, then at the end of the test period the mice are sacrificed, and their organs (an intestine, gastric, heart, liver, kidneys, spleen, and lungs) were painted using the hematoxylin-eosin. Histopathological examination was performed to determine changes in the organs microscopically. The observation was carried out for 96 hours. The number of deaths was observed and recorded at the 0th, 1st, 2nd, 3rd, 24th, 48th, 72nd, and 96th hours after dosing. This is based on the Environmental Protection Agency standard (EPA, 1998), which states that LD50 is used to determine the death of 50% of experimental animals within 24-96 hours.

Analysis

IC-50 is calculated from a linear regression equation that shows the relationship between percent of free radical scavenging and bitter melon seeds oil concentration. LD-50 value is determined based on the number of deaths in animals within 24-96 hours using probit analysis.

RESULTS AND DISCUSSION

Phytochemical screening is carried out to provide an overview of the class of compounds contained in bitter melon seeds oil. The results of phytochemical screening showed that bitter melon seeds oil contains alkaloids, triterpenoids, and flavonoids (Table I).

Determination of Total Phenolic Content

The study shows that Bitter melon seeds oil contains phenol compounds even though in a lower amount than the phenol content in grape seeds oil. The total phenol content was determined by the folin ciocalteu method using gallic acid as the standard. The standard regression equation for gallic acid is y = 0.0093x -0.0533 with a value of R2 = 0.9908 (Figure 1).

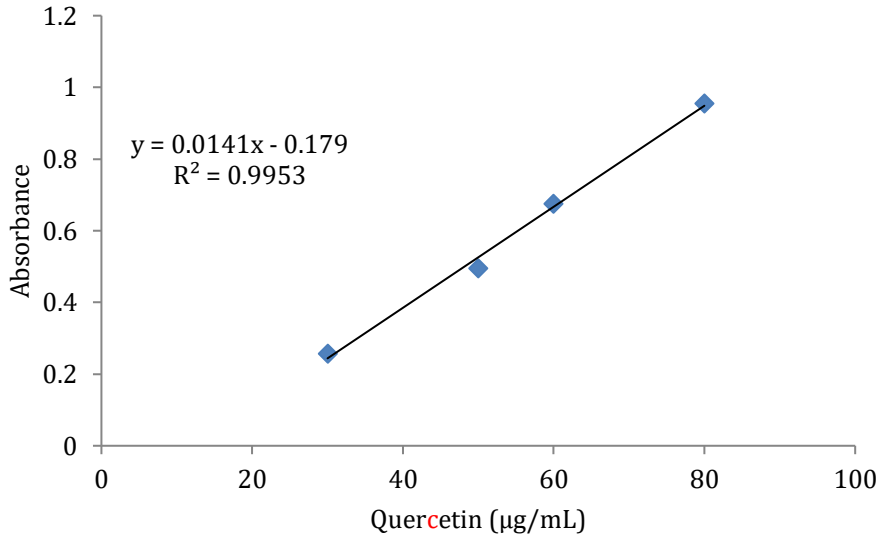


Figure 2. Total flavonoid content for standard quercetin

Table II. The Total phenolic, flavonoid and IC-50 of bitter melon seeds oil obtained from DPPH method

Sample	Total phenolic content	Total Flavonoid content	IC-50 (DPPH Method)
Bitter melon seeds oil	0.0118 ±0.0006%	0.0127±0.0004%	11310 ± 0.77µg/mL

The results showed that the total phenol content in bitter melon seeds oil was 0.0118 ±0.0006% (Table II), while in grape seeds oil, 0.0116-0.0318%. The results of the evaluation of phenol content in bitter melon seeds oil were also lower compared to the methanol extract of bitter melon fruit in dry conditions, which is 0.0257% (Lee *et al*., 2016). Despite the small phenol content, the phenol content of bitter melon seeds oil is correlated with antioxidant activity (Lutterodt *et al*., 2011) and the ability of oil conservation to oxidative reactions (Bozdagon and Mungan, 2016).

Determination of Total Flavonoids Content Flavonoids

The Flavonoids compounds in bitter melon seeds oil are analyzed because of their physiological activities such as antioxidants, antimutagenic, antidiabetic, and anticancer (Kubola and Siriamornpun, 2008). Total flavonoid levels were determined by the colorimetric method using AlCl3 and quercetin as the standard for flavonoids. The quercetin regression equation is y = 0.0141x -0.179 with a value of R2 = 0.9953 (Figure 2).

The total flavonoid in the bitter melon seeds oil was 0.0127±0.0004% (Table II); this result was lower than the flavonoid content in the methanol extract of bitter melon fruit, which was 0.0877 % (Lee *et al*., 2016).

Determination of Radical Scavenging Activity Using DPPH Method

In this study, the radical scavenging activity of bitter melon seeds oil was evaluated using the DPPH method. The DPPH method is one of the quantitative methods to find out how much the activity of bitter melon seeds oil as an antioxidant. DPPH was chosen for radical scavenging activity because it is easy, fast, simple, sensitive, and requires a small sample. The inhibition percentage of bitter melon seeds oil at various concentrations can be seen in Figure 3.

As a testing parameter is an IC-50. IC-50 is used as a parameter to determine the concentration of antioxidants that can inhibit 50% free radicals by a sample concentration (ppm) (Mailandari, 2012). The smaller the IC-50 indicates the compound has high antioxidant activity. The bitter melon seeds oil has an IC-50 value of 11.31 ± 0.77 mg / mL (Table II). The antioxidant activity of a compound can be classified based on the IC50 values. If the IC-50 of a compound is above (Molyneux, 2004). 200 ppm, its antioxidant activity

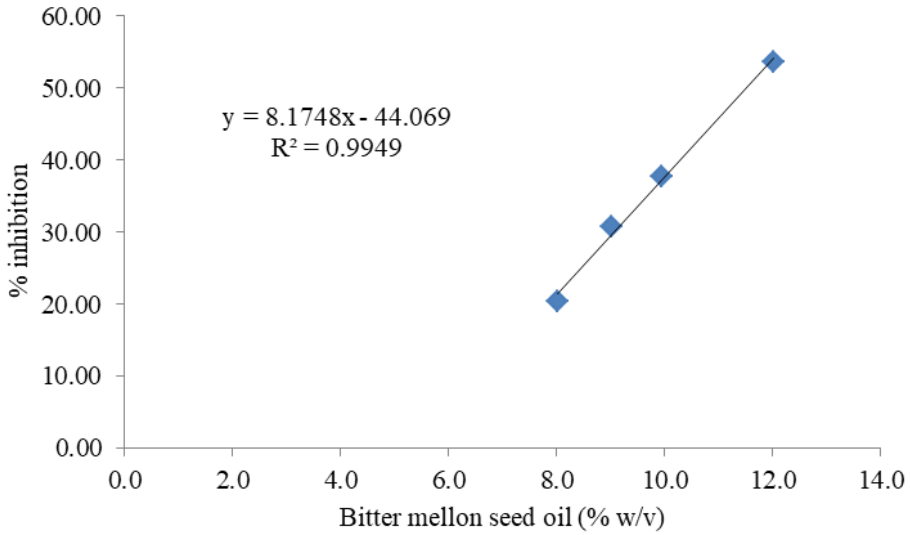


Figure 3. Percent inhibition of bitter melon seeds oil at various concentrations

Table III. Number of mice deaths during the study

Sample	Dose ml / kg	Number of Mice Deaths							
		0	1	2	3	24	48	72	96 hrs
Bitter melon seeds oil	12.5 ml / kg	0	0	0	0	0	0	0	0
	25 ml / kg	0	0	0	0	0	0	0	0
	50 ml / kg	0	0	0	0	0	0	0	0
	100 ml / kg	0	0	0	0	0	0	0	0

is very weak the results of the antioxidant activity of bitter melon seeds oil showed that their antioxidant activity was very weak. The results of antioxidant activity evaluation were in line with the results of the total phenol content and flavonoid content.

The antioxidant activity of bitter melon oil is not only produced by the content of phenols and flavonoids but also the content of Alfa-Eleostearic acid C18: 3 (9Z, 11E, 13E) and other active components such as tocopherol. The study of Anjum *et al*. (2012) showed bitter melon had vitamin E levels of 239.81-381.06 mg / g, γ-tocopherol (52.85-61.09 mg / g), and δ-tocopherol (99.74-110.07 mg / g).

Determination of Bitter Melon Seeds Oil Toxicity

Periodic observations from the 1st hour to the 96th hour, no dead mice were found at each dose level (Table III). Based on these results according to the classification of Lu (1995), the degree of bitter melon seeds oil toxicity is practically non-toxic. The LD50 value was above 15000 mg/kg because no deaths were found at a dose level of 25 ml/kg up to 100 ml/kg. The result of weighing 1 ml of the bitter melon seeds oil is equivalent to 0.923 grams; therefore, the dose is equal to 23075 m /kg - 92300 mg/kg. The dose of 92300 mg/kg is the highest dose that technically can be given to mice. Donatus and Nurlaila (1986) stated that if no dead animals were found in each dose level, then the highest dose that could technically be given to animals was considered as the LD50.

Effect of bitter melon oil on body-weight of mice

Observation of the bodyweight of mice was carried out to determine changes in the body-weight of mice during the study. The bodyweight is one of the parameters of the toxic effect. According to Lu (1995), weight loss is a sensitive index of toxic effects. The Weighing was carried out on day 0 and days 1, 2, 3, 4 of the testing period.

After the administration of bitter melon seeds oil, there was an increase in body weight at doses of 12.5 ml/kg and 25 ml/kg, whereas, at a dose of 50 ml/kg, there was a decrease in body weight at the 96th hour. Mice group dose of 100 ml/kg experienced weight loss at 24 hours and 72 hours, at 96 hours experienced weight gain. The change in body weight not show a toxic effect but shows a process of adaptation to stress after

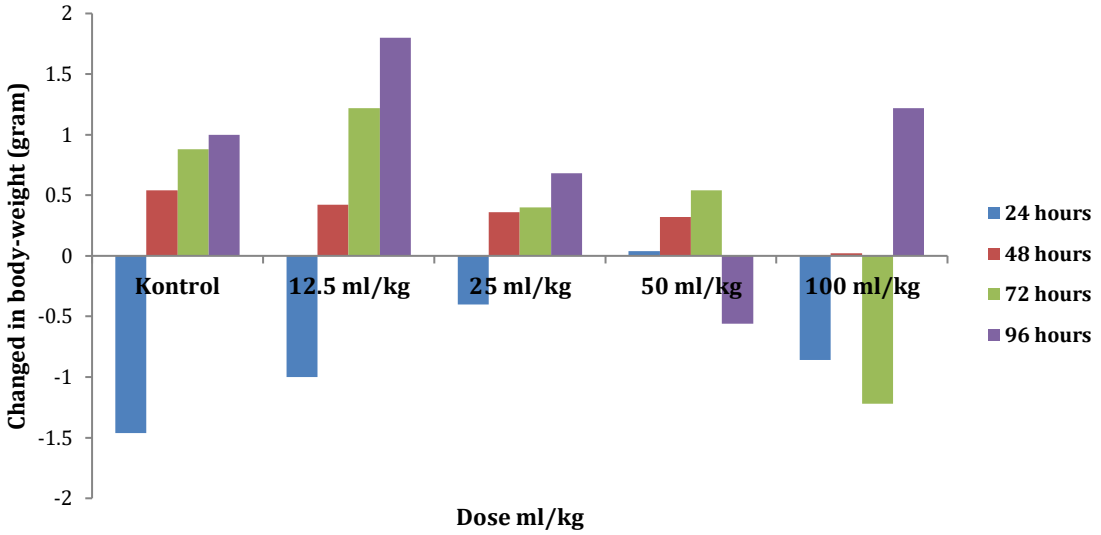


Figure 4. Changes in the weight of mice during the treatment period

Table IV. Toxic Symptoms That Occur In animals

Dose	Toxic Symptoms			
	1 hour	2 hours	3 hours	4 hours
12.5 ml / kg	Diarrhea	-	-	-
25 ml / kg	Diarrhea	-	-	-
50 ml / kg	-	Diarrhea	Diarrhea	-
100 ml / kg	Diarrhea, passive	-	Active	-

treatment. Changes in average body weight caused by the administration of bitter melon seeds oil can be seen in Figure 4.

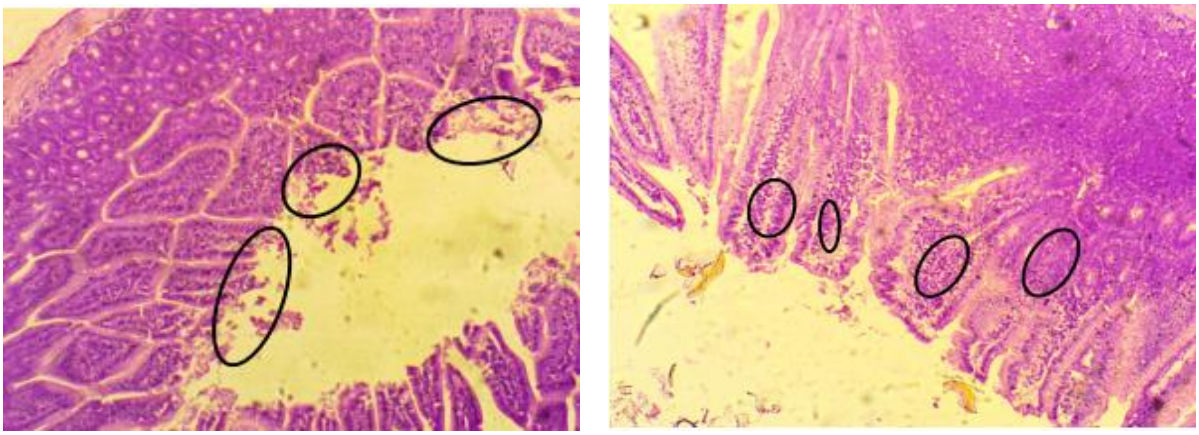
Weight loss occurs due to a decrease in food consumption after being given bitter melon seeds oil. The absorption process of fat slower than carbohydrates and protein; therefore foods that contain fat can provide a more prolonged feeling of satiety compared to foods that lack or do not contain fat (Anonymous, 2007).

Toxic Effects behavior after bitter melon Seeds oil administration

Toxic symptoms and behavior after the treatment are observed to evaluate the toxic effects that occur as a result of the administration of bitter melon seeds oil. The observation of behavior and toxic symptoms after administration of bitter melon seeds oil can be seen in Table IV.

Based on the table, it is known that in the treatment of bitter melon seeds oil, there are no toxic symptoms that attack the central nervous system, such as tremors. Toxic symptoms appear as indigestion, which is characterized by diarrhea. Diarrhea is the discharge of feces three or more times a day in the form of soft or fluid. Diarrhea itself is not a disease, but can be a symptom of several diseases and sometimes associated with pain in the abdomen that will decrease once the discharge comes out. Diarrhea occurs due to irritation in the lining of the small and large intestine, which results in reduced water absorption, therefore increasing the water that comes out with feces. Such factors as food poisoning, infection, food intolerance, malnutrition, intestinal diseases, and sometimes drugs can cause diarrhea (Tripathi, 2003). Diarrhea is reported to occur in the excess consumption of bitter melon oil in addition to the condition of diarrhea, nausea, vomiting, and stomach bleeding also occurs in consumption of bitter melon juice (Sharma *et al*., 2012).

At the end of the study, surgery was carried out for microscopic organ observation. Observation of organs aims to obtain information about the toxicity of the test substance concerning the target organ. Several organs observed were the heart, liver, lungs, stomach, intestine, kidney, and spleen. The changes that occur in the appearance of various organs cellular can be related to the content of compounds in bitter melon oil that affected the function and work of the organ.



(a) (b)

Figure 5. The cellular appearance of an intestinal organ of the mice showing epithelial erosion (a) and inflammation (b)

The results of microscopic observations found no cellular changes in the heart, lung, liver, kidney, spleen, and lungs, but in the intestinal mucosa, there are epithelial erosion and inflammation that cause low absorption of water; hence the stool is softer (Figure 5).

CONCLUSION

The results of this study indicate that the content of phenols and flavonoids in bitter melon seeds oil is quite low, as well as the effects of radical scavenging activity. The result shows that bitter melon seeds oil has very weak antioxidant activity. Although bitter melon seeds oil contains other antioxidants that were not analyzed in this study, such as tocopherol and Alfa-Eleostearic acid C18: 3 (9Z, 11E, 13E), the results showed the activity of the antioxidants are very weak. Acute toxicity study showed that the LD-50 of bitter melon oil could not be determined, although toxic symptoms such as diarrhea are found in all doses used. The bitter melon oil was categorized as a practically non-toxic compound.

ACKNOWLEDGEMENT

The authors thank IsDB Jember University for providing funding for this research.

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