Trad. Med. J., January-April 2023
Vol. 28(1), p 22-30
ISSN-p : 1410-5918 ISSN-e : 2406-9086

Submitted : 05-12-2022
Revised : 23-12-2022
Accepted : 24-01-2023

Bioassay Guided Fractionation of Ciplukan (*Physalis angulata* L.) monitored by Glucose Consumption Assay and Thin Layer Chromatography on Myoblast Cells

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ABSTRACT

Ciplukan (*Physalis angulata* Linn.) has been used by the community as an anti-diabetic drug. The antidiabetic effect is due to ingredients such as unsaturated fatty acids, alkaloids, flavonoids, saponins, polyphenols, steroids, triterpenoids, monoterpenoids, and sesquiterpenoids. Part of the fruit of *P. angulata* contains many active substances of flavonoids with the proportion of fruit extract 300 µg/ml is 84%. Therefore the exploration for compounds responsible for antidiabetic activity in *P. angulata* needs to be done to ensure empirical evidence. The purpose of this study was to find the active fraction of *P. angulata* L. which has anti-hyperglycemic properties. This study used Myoblast cells as subjects and the Bioassay Guided Fractionation method for separating compound groups through three stages of the extraction, partitioning, and fractionation processes which were monitored using TLC and the Glucose Consumption Assay test. The results showed that the chloroform extract (CHCl3) was more active in lowering glucose levels compared to the methanol extract (MeOH) (4.86% vs -8.74%). MeOH insoluble extract was more active than MeOH soluble extract (5.14% vs -8.52). The fractionation results showed that Fraction I was the most active in lowering glucose levels compared to FII, III, and IV (26.47%; 13.18%; 0.15%; 13.76%). Therefore Fraction 1 which contains a class of flavonoid compounds is a potential candidate to be developed as an antidiabetic agent.

Keywords: *Physalis angulata* Linn.; antidiabetic; Bioassay guided fractionation; glucose consumption

INTRODUCTION

Diabetes Mellitus (DM) is a worldwide disease and a major challenge for healthcare systems in all countries. Managements that have been done to prevent complications in diabetes are giving oral drugs or insulin injections. However, these drugs have many side effects, so the use of natural ingredients to overcome the problems of diabetes mellitus needs to be done. Natural materials, including medicinal plants, have many antioxidant activities which are the main source for the treatment of oxidative stress which can cause complications in diabetes (Rahimi *et al*., 2016). The development of alternative medicines for oxidative stress and related disorders is mostly based on natural ingredients. Natural ingredients containing hydrophobic compounds such as sapogenin, ursolic acid, and oleanolic, have antioxidant and hypoglycemic activities (Tripathi & Srivastava, 2006).

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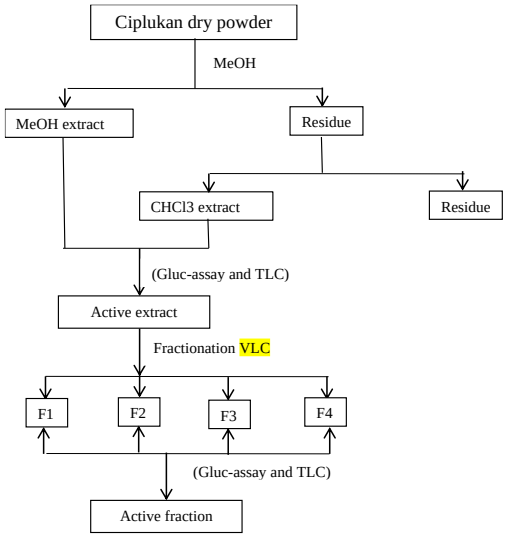
One of the plants that are widely used for a hyperglycemic conditions is ciplukan (*Physalis angulata* Linn.). Ciplukan is used by the community as an anti-diabetic drug. Ciplukan contains active compounds such as saponins (in buds), flavonoids (leaves and buds), polyphenols and fisalin (fruits), Withangulatin A (fruits and stems), palmitic and stearic acids (seeds), alkaloids (roots), chlorogenic acids (stems and leaves), tannins (fruits), cryptoxanthins (fruits), vitamin C and sugar (fruits). The fruit of the ciplukan contains citric acid, fisalin, malic acid, alkaloids, tannins, tryptoxanthin, and vitamin C. The chemical compounds contained in the ciplukan are phytosterols, chlorogenic acid, choline, ixocarpanolide, physalin A, vamonolide, withangulatin A, withanolide D, withaphysanolide and flavonoids (Sun *et al*., 2011). According to Sulistyowati (2014), the anti-diabetic effect of ciplukan is because this plant has ingredients such as unsaturated fatty acids, alkaloids, flavonoids, saponins, polyphenols, steroids and triterpenoids, monoterpenoids, and



Physalis angulata Linn. MeOH = Methanol; ChCl3 = Chloroform, TLC = Thin layer chromatography; VLC = Vacuum Liquid Chromatography

Figure 1. Bioassay-Guided Fractionation scheme for the extract's antidiabetic activity

sesquiterpenoids. *P. angulata*, especially in the fruit part, is rich in flavonoid active substances with a fruit extract percentage of 300 µg/ml is 84%. (Murali, 2013). The search for potential compounds as anti-diabetics has also been carried out by several researchers on ciplukan, including 150 mg/KgBB and 300 mg/KgBB of ciplukan ethanolic extract which significantly reduced blood glucose levels in diabetic rats (Maliangkay *et al*., 2019). Flavonoids are one of the antioxidant compounds that function to overcome or neutralize free radicals so it is hoped that damage to body cells can be inhibited by administering these antioxidants and can prevent damage to the body and degenerative diseases (Winarsi, 2007).

Bioassay-guided fractionation is the latest method for separating compounds in complex mixtures which are always monitored by activity tests and success tests for separating mixtures with TLC. This method has been successfully applied to the separation of several compounds present in natural products. Based on this background, it is necessary to study the compounds in the active fraction of *Physalis angulata* Linn in the hope of knowing where the compounds responsible for being anti-diabetic are located so that their mechanism of action can be discovered.

METHODOLOGY

Physalis angulata Linn was obtained from the Godean Region, Yogyakarta, in January 2020. Plant determination was carried out at the Department of Pharmaceutical Biology, Faculty of Pharmacy, UGM. This research has received recommendations from the Ethics Committee of FK-KMK UGM with Ref.No.: KE/FK/0241/EC/2020

Extraction, Partition, and Fractionation are presented in Figure 1

Five hundred grams of dry ciplukan powder (roots, stems, leaves, and fruits) was extracted by maceration using 1 liter of methanol accompanied by stirring for 24 hours at room temperature. Filtering was carried out using a Buchner funnel and the dregs were macerated twice again in the same way and filtered. The filtrates obtained were combined and evaporated to obtain a thick methanol extract (A). The residue was aerated until it did not smell of methanol then it was macerated again, in the same way, using chloroform solvent and then filtered. The filtrates obtained were combined and evaporated to obtain a thick chloroform extract (B). Extraction success was monitored by thin-layer chromatography and glucose consumption assay.

Three grams of active extract (B) was then partitioned using methanol centrifuged at 5000 rpm [Centrifuge (Hitachi 18PR/5, Automatic high speed refrigerated)] for 10 minutes, to obtain soluble MeOH extract (extract B1) and MeOH insoluble extract (extract B2). Partition success was monitored by TLC which was indicated by the absence or as little as possible of the similarity of

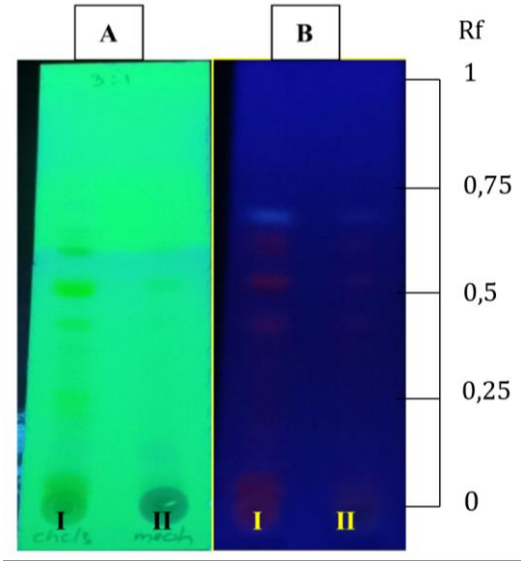


Figure 2. TLC profile from P. angulata extraction

I = CHCl3 extract, II = MeOH extract, A = 254 nm UV light; B = 366 nm UV light. Stationary phase = silica gel 60 F254; Mobile phase = wash benzene : ethyl acetate (3:1, v/v)

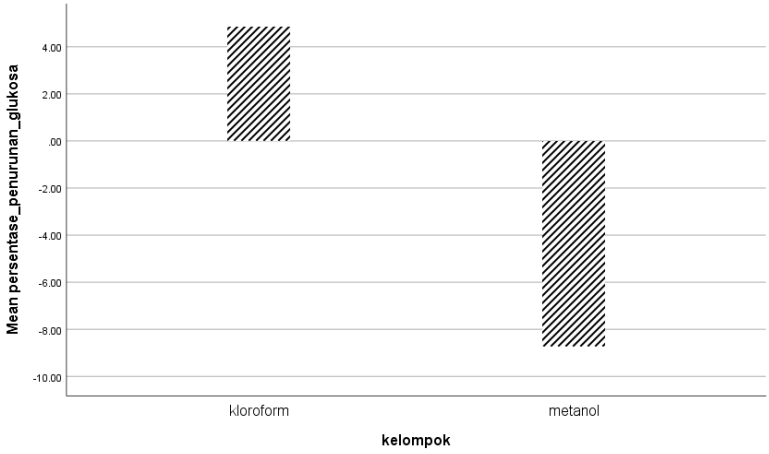


Figure 3. Percentage of glucose reduction in the CHCl3 vs MeOH *P. angulata* extract group

the spots between the two extracts, then each extract was tested by a glucose consumption assay.

The active extract (B2) was then fractionated using liquid chromatography modified using vacuum (Coll and Bowden, 1986; Pelletier *et al*., 1986) with preparative grade GF254 silica gel stationary phase. The mobile phase was used with increasing polarity, respectively; n-hexane (100%), n-hexane: ethyl acetate (9:1 v/v), n-hexane: ethyl acetate (8:2 v/v), n-hexane: ethyl acetate (7:3 v/ v), n-hexane: ethyl acetate (6:4 v/v), n-hexane: ethyl acetate (5:5 v/v), ethyl acetate (100%) and finally with chloroform: methanol (1:1 v /v). The eluate obtained was collected in a porcelain cup and evaporated to dryness. Each fraction was examined by TLC and the fractions which showed similar spots on the chromatogram were combined and evaporated. The combined fractions obtained were weighed, then tested by glucose consumption assay and TLC.

Antihyperglycemic Test with Glucose Consumption assay Method (ab65333 Glucose Assay Kit colorimetric)

Myoblast cells (C2C12) were cultured at 10.000 cells/well in 96 wellplates. DMEM + 10% FBS media from Myoblast cells was replaced with 2% DMEM + Horse Serum or 2% FBS every 2 days

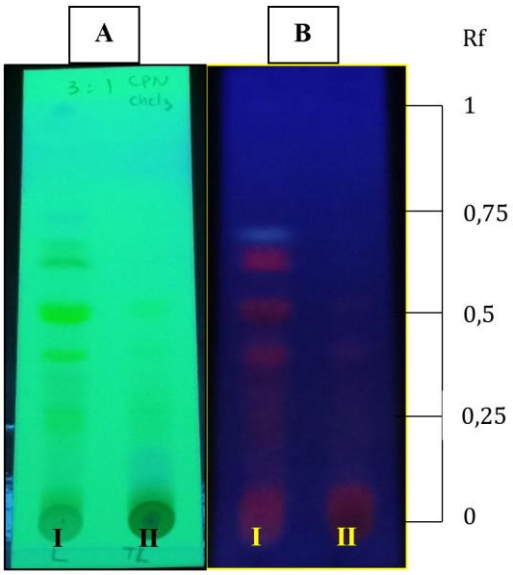


Figure 4. TLC profile of *P. angulata* partitions

I = soluble fraction, II = insoluble fraction, A = 254nm UV light; B = 366 nm UV light; Stationary phase = silica gel 60 F254; Mobile phase = wash benzene : ethyl acetate (3:1, v/v)

until they became myotubes (± 5 days). After becoming myotubes, the cells were treated with samples (results of extraction, partitioning, and fractionation of ciplukan) continuously with the required concentration, then incubated for 48 hours. After 48 hours, a glucose consumption test was carried out using GOD-PAP by taking 1 µL Myoblast cell treatment medium added with 100 µL GOD-PAP solution, then incubating for 20 minutes at room temperature. Glucose (100 mg/dL) and H2O were used for standard and blank respectively. Read the absorbance on the ELISA reader with a wavelength of 490 nm.

RESULT AND DISCUSSION

The initial step in this bioassay-guided fractionation was the separation of polar and non-polar compound groups. Separation of non-polar compound groups was carried out by maceration of ciplukan powder using chloroform (CHCl3) solvent which can extract non-polar to semi-polar compound groups, while Methanol (MeOH) solvent can extract polar to semipolar compound groups. As seen in Figure 2, the separation of the extracts was successful, as evidenced by the fact that no/slight duplication of spots was detected when viewed at UV 254nm or UV366nm. The percentage of glucose reduction was presented in Figure 3.

The results of the antidiabetic mellitus activity test using the glucose consumption assay method can be seen in Figure 3, showing that the CHCl3 extract was more active in lowering glucose levels compared to the MeOH extract. This proves that the candidate for antidiabetic agents lies in a class of compounds that are non-polar to semi-polar. To further ascertain where the active compound is located, further separation must be carried out, through the partitioning of the compound from the active extract. Based on this explanation, it can be temporarily concluded that the CHCl3 could lower glucose levels higher than the MeOH extract. In other words, the more active extract was the CHCl3 extract with the percentage of glucose reduction relative to the negative control, which was 4.86%. methanol extract has a glucose reduction percentage of -8.74% relative to the negative control. These results indicated that the glucose consumed by the cells was more in the group of cells that were given ciplukan chloroform extract.

When viewed from the TLC profile of the partition results from the active extract (CHCl3) it showed that the partitioning of the MeOH soluble compound group has a different profile from the MeOH insoluble compound group. The solid-liquid partition using MeOH solvent was intended to separate/clean a group of compounds that are semipolar in the CHCl3 extract (Figure 4).

In Figure 5 it was stated that the partition results in insoluble MeOH having a higher glucose-

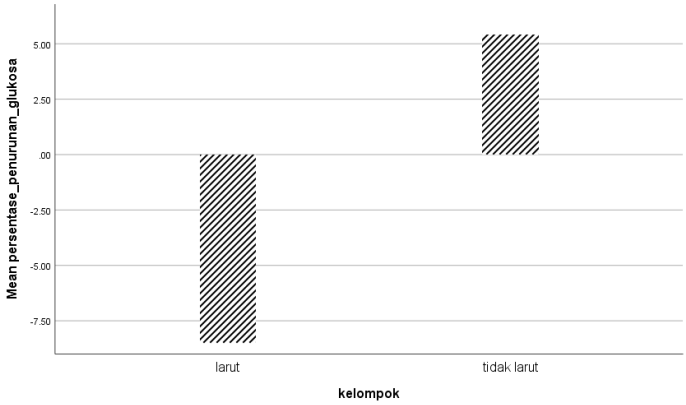


Figure 5. Percentage of glucose reduction in the soluble vs insoluble *P. angulata* partition group

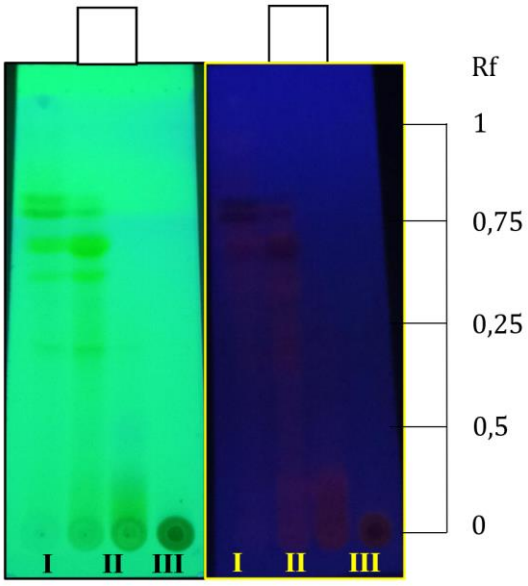


Figure 6. TLC profile of *P. angulata* fractionation

I = fraction 1, II = fraction 2, III = fraction 3, IV = fraction 4, A = 254 nm UV light; B = 366 nm UV light. Stationary phase = silica gel 60 F254; Mobile phase = wash benzene : ethyl acetate (3:1, v/v)

lowering activity compared to partitions that were soluble in MeOH. Therefore, the results of partitions that were not soluble in MeOH will be carried out for further processes, namely fractionation. The results of this activity indicated that the MeOH insoluble partition showed a higher percentage of glucose reduction, 5.14%, compared to the soluble partition -8.84%. These results indicated that the glucose consumed by the cells was more in the group of cells that were given insoluble MeOH partitions of ciplukan.

The last step in separating this group of compounds was by fractionating using solvents with different polarity gradients in the hope that the active partition results, the insoluble MeOH partition, can be fractionated according to the polarity of the compounds. The results of the fractionation of the insoluble MeOH fraction are presented in Figure 6. The results of the fractionation showed that the compounds contained in the active partition results have been well fractionated as shown in Figure 6.

The activity test results for the 4 fractions showed that fraction I was the most active fraction compared to fractions 2, 3, and 4. From Figure 7, fraction 1 showed the non-polar compound group towards semi-polar. To ensure which compound class was contained in fraction I can be seen in Figure 8. For the time being the conclusion at this fractionation stage was that the most active

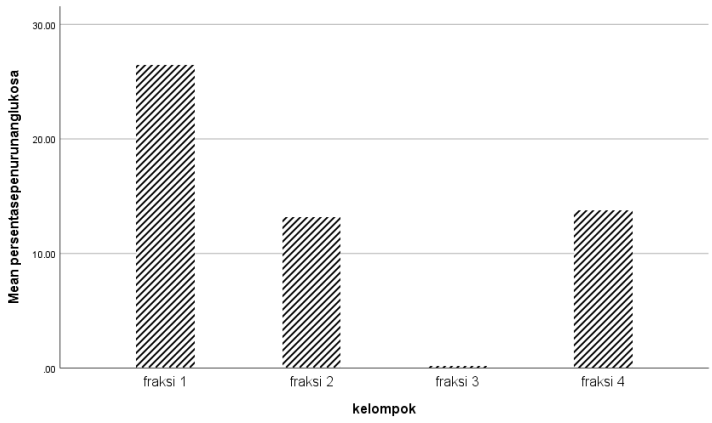


Figure 7. Percentage of glucose reduction of *P. angulata* fraction 1, 2, 3, 4

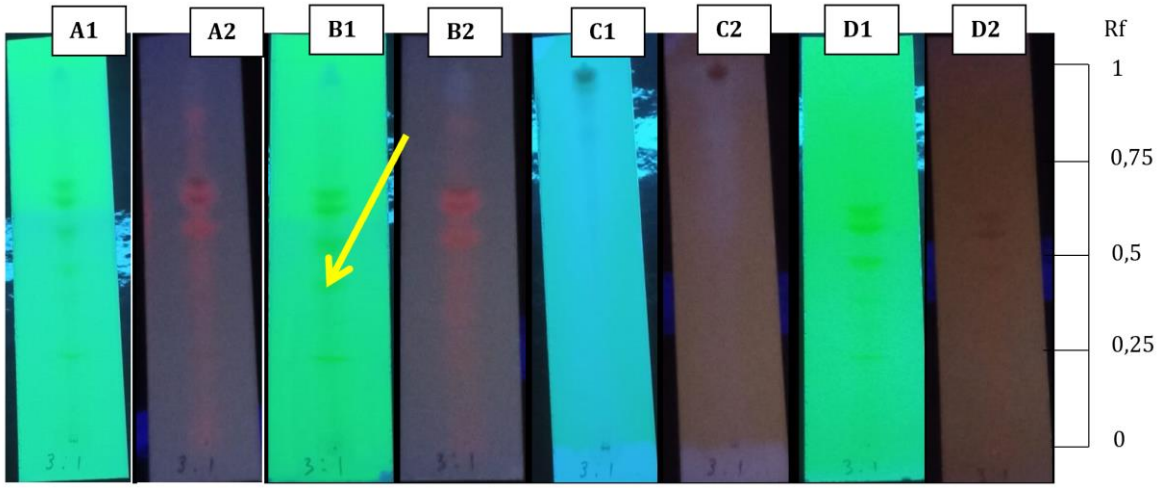


Figure 8. Chromatogram profile of *P. angulata* Fraction 1

A1= under UV light 254 nm; A2= under UV light 366 nm; B1= after spraying with citroborate under UV light 254 nm; B2= after spraying with citroborate under UV light 366 nm; C1= after spraying with cerium sulfate under UV light 254 nm; C2= after spraying with cerium sulfate under UV light 366 nm; D1= after spraying with dragendorf under UV light 254 nm; D2= after spraying with dragendorf under UV light 366 nm. Stationary phase = silica gel GF254; Mobile phase = wash benzene : ethyl acetate (3:1, v/v)

fraction was fraction 1 with the percentage of glucose reduction relative to the negative control which was equal to 26.47% followed by fraction 4, fraction 2, fraction 3. These results indicate that the most glucose consumed by cells was in the group of cells that were given ciplukan fraction 1.

As a summary of the activity tests from extraction, partitioning and fractionation is presented in Table 1. From Table 1 it can be seen how the activity of each step of separating compound groups using the bioassay-guided fractionation method. The bioassay-guided fractionation method has been used previously to test the anticancer activity of compounds isolated from *N. indicum* leaves (Wahyuningsih *et al*., 2017), detecting the presence of the active fraction of *T. diversifolia* (Hemsley) A Gray which has antiplasmodial potential (Syarif *et al.*, 2018). This method is widely used in the early stages of isolated compounds from natural materials, such as tagitinin C from T. *diversifolia* (Wahyuningsih *et al*., 2015) and Zerumbone from *Zingiber zerumbet* (Murini *et al*., 2018).

From Table I it can be seen that fraction 1 was the most active fraction with a glucose reduction percentage was 26.47% compared to the other fractions, then identification was carried out qualitatively using TLC with certain stationary and

Table I. The percentage of glucose reduction for the Glucose Consumption Assay of all extracts using Bioassay guided Fractionation.

Extracts	% Glucose Reduction
MeOH	-8,74
CHCl3	**4,86**
MeOH soluble	-8,84
MeOH insoluble	**5,14**
Fraction I	**26,47**
Fraction II	13,18
Fraction III	0,15
Fraction IV	13,76

mobile phases. The results of the characterization of the compound groups contained in fraction 1 are presented in Figure 8.

The TLC results of the extraction and partition showed that the active ingredients that could lower glucose levels were nonpolar compounds. In further assistance, identification of fraction 1 with TLC found that the class compounds contained in the active fraction in the group of the majority of Flavonoid compounds group, as evidenced by the citroborate visual reagent, both seen using UV 254 or UV 366 (B1 and B2). Spots were visible indicating the flavonoid group. These results are following previous research that the highest content in ciplukan is Flavonoids besides alkaloids and saponins (Sun *et al*., 2011). The anti-diabetic effect of ciplukan is because this plant contains chemical compounds such as unsaturated fatty acids, alkaloids, flavonoids, saponins, polyphenols, etc. (Sulistyowati, 2014).

CONCLUSION

The results of this study indicated that the CHCl3 extract had better glucose-lowering activity than the MeOH extract (4.86 vs -8.74). MeOH insoluble extract had better glucose-lowering activity than MeOH soluble extract (5.14 vs -8.84). Fraction I had the best glucose-reducing activity compared to the other fractions. Therefore fraction 1 which contains the majority of the Flavonoid compound class is a potential candidate to be developed as an antidiabetic mellitus agent.

ACKNOWLEDGEMENT

The author would like to thank FKKMK UGM for providing a Community Fund Grant for Lecturer-Student Scheme 2020 to finance this research.

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