

Antioxidant and HPTLC Study of Black Glutinous Rice Extract from South Sulawesi Indonesia

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ABSTRACT

Black glutinous rice (BGR) is one of widely used materials for Buginese and Makassarnese people as traditional medicine ingredients and food compositions. It is a daily-consumed mixture for people with health problems such as diabetes mellitus, asthma, post-partum treatment, and gastritis. It has antioxidant effects due to its unique natural colorants, anthocyanin which been claimed as the main cause of its early-mentioned medicinal effectiveness. In order to have clear scientific evidence, this research was aimed to determine HPTLC profile and antioxidant activity of BGR. Black glutinous rice was extracted with acidic methanol, ethanol, and acetone solvents, then the analysis of anthocyanin was determined by the pH-differential and high-performance thin-layer chromatography (HPTLC) method. The antioxidant activity was investigated using 2,2-diphenyl-1-picryl hydrazine (DPPH), Nitric Oxide (NO) radicals scavenging, and β -Carotene bleaching (BCB) assay. The highest total anthocyanin content was found in methanolic extract (0.166% b/b), followed by ethanolic extract (0.126% b/b) and acetonic extract (0.076% b/b). The antioxidant activity was inversely significant correlated ($p < 0.01$) with the anthocyanin content; the highest antioxidant was observed in acetone extract with IC_{50} of 14.49 μ g/ml for DPPH, 48.97 μ g/ml for NO and 131.97 μ g/ml for BCB assay. Furthermore, the HPTLC profile revealed that the primary compound in all BGR samples is cyanidin-3-o-glycoside, but there is another compound content that contributes to the higher activities of the BGR acetone extract. The antioxidant activity of black glutinous rice (*Oryza Sativa* Linn. var *glutinosa*) may be due to the presence of the anthocyanin and other nonpolar compounds.

Keywords: black glutinous rice, South Sulawesi, antioxidant, HPTLC

INTRODUCTION

Black glutinous rice (*Oryza sativa* Linn. var *glutinosa*) is a variety of widely consumed rice in South Sulawesi. It has high antioxidant activity because of its high anthocyanin content¹⁻⁶. Compared to other pigmented rice varieties: black rice, and red rice, the black glutinous rice has the highest anthocyanin content^{3,5,7}. Anthocyanin is a plant pigment providing red, blue, and purple colors in various plants, which is included in flavonoid group. Some research shows that the anthocyanin in black glutinous rice possesses antioxidant and radical scavenger activities, antimutagenic, anti-inflammation, anti-hyperlipidemia, and anticarcinogenic. Anthocyanin extract has been reported in improving eyesight sharpness, decreasing lipid content, increasing insulin secretion, protecting to the liver, and also having vasoprotective effects^{2,5-12}. Extracting anthocyanin from black glutinous rice is generally involving several solvents such as methanol, ethanol, and acetone which contain acid to keep the anthocyanin stability^{7,10,12}. Based on the above illustration, this study has directed to research the antioxidant activity of BGR from South Sulawesi, Indonesia. TLC and HPTLC profiling have been performed to standardize and to ascertain the possible compound

which are responsible for the extracts activities. Anthocyanin cyanidin-3-glucoside was used as a marker substance. Results from this study might give the preliminary *in vitro* information to support the capability of BGR extract from Indonesian glutinous rice as a pharmaceutical and nutraceuticals.

MATERIALS AND METHODS

Chemicals and solvents

Chemicals such as DPPH, β -carotene, linoleic acid, and nitric oxide were purchased from Sigma-Aldrich Company, St. Louis, Missouri, USA. TLC plates, solvents and other chemicals (analytical grade quality) were obtained from E. Merck.

Plant material

Black glutinous rice (*Oryza sativa* L.) was collected from South Sulawesi province.

Extraction of Anthocyanin¹¹

BGR was extracted utilizing different organic solvents, namely acidified methanol, acidified ethanol and aqueous acidified acetone. Re-extraction process was conducted until colorless. The subsequent extracts were evaporated by

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Table 1: The extraction yield and total anthocyanin content of the black glutinous rice extracts

	Methanol Extract	Ethanol Extract	Aceton Extract
The extraction yield (% w/w)	2.29	2.25	2.69
Total anthocyanin content (% w/w) ^a	0.166±0.015	0.126±0.016	0.077±0.005

^aMean ± standard deviation for triplicate experiments.

rotary evaporator at 50°C. They were weighed in determining yield of obtained extract by the following equation¹¹.

$$\text{Yield (\%)} = \frac{\text{weight of BGR extract after evaporation}}{\text{weight of BGR powder before extraction}} \times 100\%$$

Determination of Total anthocyanin content

The total anthocyanin content was defined through the pH differential method^{10,11}. UV-Vis spectrophotometer (Agilent) with 1 cm path length disposable cuvettes was used for spectral measurements at 509 and 700 nm. The color substance was figured as cyanidin-3-glucoside, utilizing an extinction coefficient of 29600 L cm⁻¹ mg⁻¹ and molecular weight of 448.8.

TLC-autography with DPPH free radical spraying

Antioxidant spots were recognized on TLC plate by spraying with DPPH radical. Antioxidants diminish the radical, creating white spots on a purple background¹³.

DPPH free radical scavenging activity

The free-radical scavenging activity of BGR extracts was measured by reduction in absorbance of the methanolic solution of DPPH by a microplate spectrophotometric method as previously described¹⁴. Briefly, DPPH solution (75 µl, 240 µM) and a 100 µg/ml solution of black glutinous extract (20, 40, 60, 80 and 100 µl, respectively) were added to each well of the microplate and mixed. The mixture was shaken vivaciously and left to stand at 37 °C for 30 min in the dark. The absorbance was subsequently measured at wavelength 515 nm utilizing a microplate reader. Inhibition (%) of free radical (DPPH) in percent was resolved as:

$$\% \text{ inhibition} = \frac{A_0 - A_t}{A_0} \times 100\%$$

Where A₀ is the absorbance of the control reaction containing all reagents aside from the test sample, and A_t is the absorbance of the BGR extract. Tests were conducted in triplicate. Ascorbic acid (2.5, 5.0, 7.5, 10 and 12.5 µg/ml) was used as the positive control. By then, % inhibitions were plotted against particular concentrations of the extract and from the calculated graph IC₅₀.

Nitric oxide scavenging activity

Nitric oxide scavenging activity was evaluated spectrophotometrically. Sodium nitroprusside (5 mmol/L) in phosphate buffer saline pH 7.4, was mixed with different concentrations of the extract (250–2500 mg/mL) prepared in methanol and incubated at 25°C for 30 min. After incubation, 1.5 mL of the incubated mixture was picked up and debilitated with 1.5 mL of Griess reagent (1% sulphanilamide, 2% phosphoric destructive and 0.1% N-1-naphthylethylene-diamine dihydrochloride). The absorbance of the chromophore encircled in the midst of diazotization of the nitrite with sulphanilamide coupling with N-1-naphthylethylene diamine dihydrochloride was

measured at 550 nm¹⁵⁻¹⁷. Ascorbic acid, used as a positive control treated comparatively with Griess reagent.

β-Carotene bleaching (BCB) assay

The antioxidant activity of the rice extract was evaluated by β-carotene-linoleic acid bleaching method described by Wang et al. (2010) and Farag et al (1989) with slight modifications^{13,17}. The linoleic acid (25 µL) and Tween-40 (200 mg) were mixed in the β-carotene solution (0.5 mg of β-carotene go into solution in 1 mL of chloroform). Chloroform was then eliminated utilizing a rotary evaporator at 50 °C. Distilled water (50 mL) was added and the mixture was vigorously shaken for several minutes until the emulsion of β-carotene-linoleic acid was formed. The above β-carotene - linoleic acid - Tween 40 mixture (90 µL) and the extracts solution (10 µL, concentrations from 100µg/mL to 500µg /mL) in methanol solution were added to each well. A proportionate quantity of methanol was applied as the control. The microplates were then placed in an incubator at 50 °C for 2 h along with α-tocopherol (10 µL, concentrations from 2.0 µg/mL to 10 µg /mL) as the positive control. The absorbance of the solution was then measured at wavelength 490 nm using a microplate spectrophotometer. The percentage (%) of β-carotene bleaching inhibition of each sample was calculated as¹⁸.

BCB activity

$$= \left(1 - \left(\frac{A_{\beta\text{-carotene after 2 h assay}} - A_{\beta\text{-carotene}}}{A_{\text{initial blank}} - A_{\text{blank after 2 h assay}}} \right) \right) \times 100\%$$

Where A_{β-carotene after 2 h assay} is the absorbance of the sample with β-carotene-linoleic acid mixture after the two-hours period of incubation, and A_{initial β-carotene} is the absorbance of the initial mixture. All tests were conducted in triplicate. The IC₅₀ value (50% inhibitory concentration) was determined by probit analysis of concentration log data with probit of free radical binding percentage.

β-carotene bleaching rate was calculated using a formula:

$$R = \frac{\ln \left(\frac{a}{b} \right)}{t}$$

Where R = β-carotene bleaching rate, a = absorbance at t=0, b = absorbance at t=120, t = 120 minutes.

HPTLC (High-Performance Thin Layer Chromatography) Profile

Each extract was analyzed by High-Performance Thin Layer Chromatography (HPTLC). Various dissolvable systems have been tried in obtaining the optimum determination, which was acquired in the dissolvable methanol-water in the proportion of 8:2. Samples were applied on pre-coated silica gel 60 F254 aluminum sheets (10 x 20 cm). Then, the obtained chromatograms were scanned by densitometer at 254 nm. The R_f values and fingerprint details were noted by WIN CATS software^{18,19}.

RESULT AND DISCUSSION

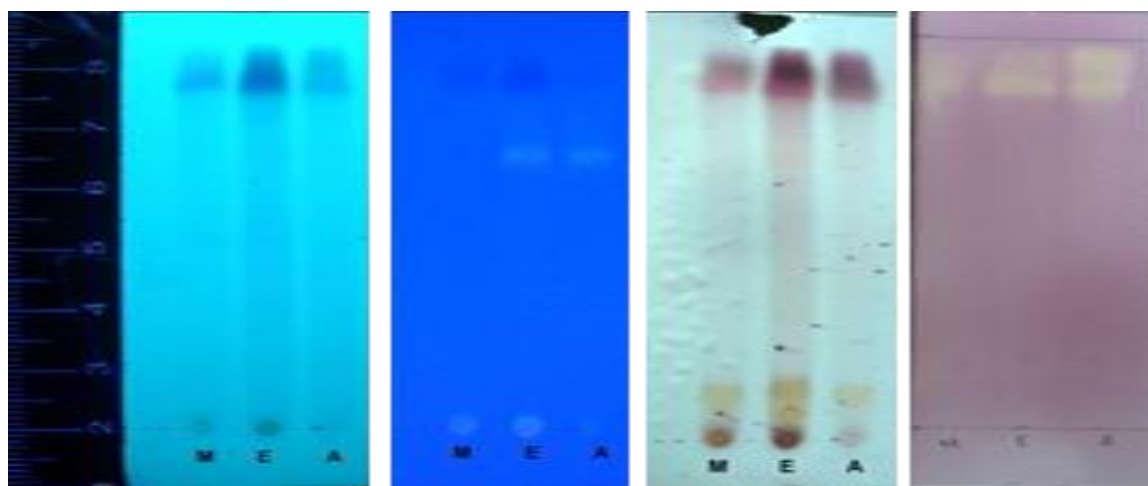


Figure 1: Profile of Thin Layer Chromatogram of Black glutinous rice extracts

Table 2: The antioxidant activities of the black glutinous rice extracts

Assay	Samples*			
	Methanol Extract	Ethanol Extract	Aceton Extract	Positive control**
DPPH scavenging (IC ₅₀ in µg/mL)	47.516±1.933	23.326±2.850	14.498±0.291	2.137±0.157
NO scavenging (IC ₅₀ in µg/mL)	256.657±3.474	291.273±13.286	48.973±2.312	7.111±0.201
β-Carotene bleaching inhibition (IC ₅₀ in µg/mL)	405.96 ± 55.34	351.62 ± 19.33	131.97 ± 19.43	5.51 ± 0.39
β-Carotene bleaching rate (menit ⁻¹) ***	0.2601±0.0308	0.1826±0.0047	0.1165±0.0039	0.1303±0.0025

* Mean ± standard deviation for triplicate experiments.

** The positive controls for DPPH and NO were ascorbic acid, and for BCB-assays was α-tocopherol.

***The concentration of aliquot sample are 300 µg/ml and 10 µg/ml for BGR extract and α-tocopherol, respectively.

Extraction of anthocyanin compounds performed in acidic condition with the addition of 0.01% HCl because anthocyanins more stable in acidic than neutral or alkaline. Determination of total anthocyanin content is performed by the pH-differential method. This method is a rapid and easy procedure for the quantification of monomeric anthocyanins, even in the presence of polymerized degraded pigments and other interfering compounds. It is based on the structural transformation of the anthocyanin chromophore as a function of the pH, which can be measured using optical spectroscopy. The stained oxonium form prevails at pH 1.0 and the uncolored hemiketal form at pH 4.5^{10,11,20}. The polar character of anthocyanin molecule takes into consideration its solvability in a wide range of solvents: methanol, ethanol, and acetone^{10,11}. Extraction of black glutinous rice using methanol, ethanol and acetone in acidic conditions shows different anthocyanin content of the extracts. The total anthocyanin content was decreased in the following order: methanolic extract > ethanolic extract > and acetonic extract (Table 1): Free radicals, shaped by different natural chemicals and also by endogenous digestion system, can lead transformation and chromosomal damage, oxidative damage to DNA, cell thiols oxidation, and ‘stealing’ hydrogen molecules from unsaturated fats to start the peroxidation of layer lipids. This harm has been widely accepted to be the fundamental reason for diseases such as cardiovascular issues and immune system decline^{21,22}. In

this research we examined the antioxidant activity of BGR extracts with 3 different methods, DPPH, NO, and BCB assay.

The DPPH

DPPH is generally steady nitrogen centered free radical that effectively acquires an electron or hydrogen radical to wind up a stable diamagnetic atom. DPPH radicals respond to suitable reducing agents as a result of which the electrons become matched off shaping the relating hydrazine. Thus, the solution depletes color stoichiometrically relying upon the quantity of electrons taken up²³.

The TLC autography results of the extracts are presented in Fig 1.

(Adsorbent =RP-18 silica gel GF₂₅₄, Mobile Phase = Methanol-water 8:2) seen at I = UV 254, II = UV 366, III = H₂SO₄ and DPPH 0.2%). M= methanol extract, E= ethanol extract, A=Acetone extract. The thin layer chromatography (TLC)-autography study of BGR extract (Figure 1) showed two spots (R_f = 0.87 and R_f = 0.93), which entrap DPPH radical (antioxidant reduce the DPPH radical, producing yellow spots on a purple background). Acetone extract showed as the yellow stains were the brightest compared to methanol and ethanol extracts. The 2nd spot (R_f = 0.93) on acetone extract-TLC was not clearly observed on ethanol and methanol extracts. Our results in the quantitative study showed that the acetone extract (IC₅₀14.49µg/ml) displayed significantly higher DPPH scavenging activity compared to methanol extract

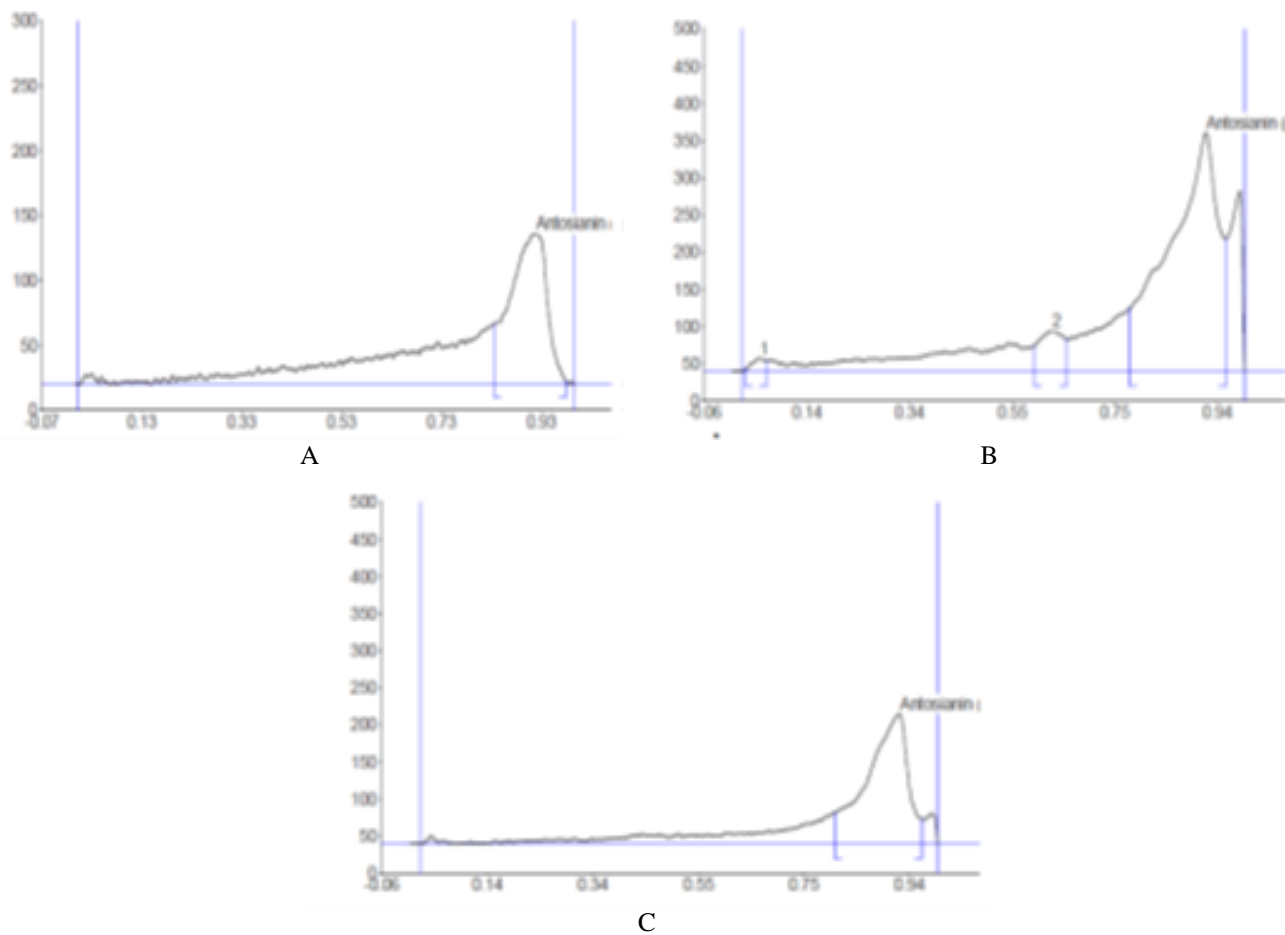


Figure 2: HPTLC of Black glutinous rice extracts using cyanidin-3-glucoside as a marker compound (a) Methanol extract (b) Acetone extract (c) Ethanol extract. Adsorbent = silica gel 60 F₂₅₄ RP-18, Mobile Phase = Methanol-water 8:2, detected at UV 254nm)

(IC₅₀ 47.52 μg/ml) and ethanol extract (IC₅₀ 23.33 μg/ml), but two times lower than the ascorbic acid (IC₅₀ 7.11 μg/ml) activity as a positive control. This result has been similarly reported by Chakuton (2012) and Park (2008)^{1,24}. Antioxidant activity of BGR extracts is presented in Table 2. The data in Table 2 also showed that all of the extracts effectively scavenge the nitric oxide radical. Nitric oxide (NO) is a vital mediator which is released by endothelial cells, macrophages, and neurons. It is a potent mediator of physiological processes such as smooth muscle recovering, neuronal signaling, inhibition of platelet aggregation and regulation of cell-mediated toxicity. Oxygen reacts with the excess NO to generate nitrite and peroxy nitric anions, which act as free radicals^{15,25,26}. In the present study, the nitric produced by the incubation of solutions of sodium nitroprusside in standard phosphate buffer at 25° C was reduced by BGR extracts. Methanolic and ethanolic BGR extracts at concentration 300 μg/ml were able to inhibit almost 50-55% of nitric oxide radicals. Acetone extract of BGR could achieve the same inhibition at 60 μg/ml. Meanwhile, ascorbic acid as a positive control possessed the inhibition potency at concentration 3.0 μg/ml. Probably, antioxidant is responsible for this activity through competition with oxygen to react with nitric oxide, which subsequently restraining the production of nitrite^{25,26}.

However, here we found that NO free radicals scavenging activity of the extracts was approximately 5 times lower than its DPPH scavenging activity.

The β-carotene bleaching (BCB)

The β-carotene bleaching (BCB) assay was conducted using a model lipid substrate (linoleic acid), in emulsion system. Linoleic acid was used as a radical source. Linoleic acid is an unsaturated acid with double bonds, thus it would be oxidized in the presence of oxygen during incubation at the temperature of 50°C producing peroxy radical and other free radicals. Subsequently, they would attack β-carotene double bonds, so that the orange color of β-carotene being bleached.² The IC₅₀ of BCB of acetone extract, ethanol extract, and methanol extract were 131.97 ± 19.43, 351.62 ± 19.33, 405.96 ± 55.34 μg/ml, respectively. The BCB-activity of the extracts was approximately 10 times lower than its DPPH scavenging activity. β-carotene bleaching occurred quickly without an antioxidant (3.0324 ± 0.447 minute⁻¹). With the presence of an antioxidant in BGR extract, the β-carotene bleaching rate was deterred. Flavonoid compounds such as anthocyanin would give its hydrogen atoms to the radicals so the radicals became stable^{2,5,27}. This bleaching inhibition could be observed through microplate reader at a wavelength 470 nm²⁶. α-tocopherol showed the highest

bleaching rate inhibition ($0.1303 \pm 0.0025 \text{ minute}^{-1}$) at $10 \mu\text{g/ml}$ concentration, followed by acetone extract ($0.1165 \pm 0.0039 \text{ minute}^{-1}$), ethanol extract ($0.1826 \pm 0.0047 \text{ minute}^{-1}$), and methanol extract ($0.2601 \pm 0.0308 \text{ minute}^{-1}$). Statistical analysis results using Oneway ANOVA method, continued with Tukey method showed the highly significant difference ($p < 0.001$) between β -carotene bleaching rate in the blank and the extracts. However, the bleaching rate among the three extracts was insignificantly different ($p > 0.5$). Based on the three different methods: DPPH, NO, and BCB assay, it may be concluded that the antioxidant-activity of BGR extracts is originally provided by their electron transfer (ET) and hydrogen donating ability²³. Unpredictably, according to the result of Pearson correlation analysis, the anthocyanin concentration of BGR and its antioxidant activity were suggested has negatively correlation ($p < 0.05$). This suggested that the lowest anthocyanin contained in the extract has the highest antioxidant activity that may be caused of other compounds having strong antioxidant activity, also presence in the acetone extract. This phenomenon has been similarly reported by Veliogluet al. (1998) suggested that plants with phenolics (mainly anthocyanins), may not has a significant correlation between antioxidant assay results²⁸. Therefore, future isolation and spectroscopic characterization will provide more data on the chemical identity of the other constituents in the extracts. The HPTLC study of methanolic, ethanolic and acetone extracts of BGR was carried out along with the anthocyanin marker cyanidine-3-glucoside compounds to ensure the presence of active ingredients in all extracts. The TLC and HPTLC profiles of the extracts are presented in Fig 2. Some investigations have shown that herbal medicines extracts are still conflicting as far as natural action and restorative effects. This irregularity might come because of insufficient standardization and poor portrayal of the assessed extracts. Therefore, in this study, we standardized the extraction methods and characterized the plant extract to provide a scientific reason for the utility of the active extract. The anthocyanin content of the extracts was estimated by HPTLC using cyanidin-3-glucoside as a marker compound, which showed Rf value of 0.92-0.93. The HPTLC figure for the methanolic, ethanolic and acetone extracts of BGR sample showed the same one peak at Rf values 0.93. The other peaks were obtained in acetone extract at Rf value of 0.05 and 0.62, indicating the presence of the other phyto constituents, which may be responsible for the activity of the acetone extract. The other valuable compounds in the black glutinous rice may be gamma-oryzanol, vitamin E complex, tocotrienols, and β -sitosterol^{1,29,30}.

CONCLUSIONS

The acetonic extract has the highest antioxidant activity, followed by ethanolic extract and methanolic extract. The activity of black glutinous rice (*Oryza Sativa* Linn. var *glutinosa*) may be due to the presence of the anthocyanin and the other nonpolar compounds. Further characterization of these extracts by applying more advanced separation and purification techniques are

necessary to find out the exact chemical compounds and their relation to the activity.

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