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ANTIBACTERIAL, RADICAL- SCAVENGING ACTIVITIES AND CYTOTOXICITY PROPERTIES OF *PHALERIA MACROCARPA* (SCHEFF.) BOERL. LEAVES IN HEPG2 CELL LINES

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ABSTRACT

Keywords:

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Antibacterial, and radical scavenging activities of four crude extracts of *Phaleria macrocarpa* (Scheff) Boerl.) leaves extracts and its active crude cytotoxic properties in HepG2 cells were investigated. The susceptibility bacteria tests were performed by the Mueller Hinton agar well diffusion method. The Radical scavenging activity was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) method, while antioxidant potency was tested by DPPH TLC bioautographic assay. The cytotoxic was measured by the MTT assay against human hepatoma cell lines (HepG2). Crude extract MMD showed an equally good activity against *Pseudomonas aeruginosa*, and strong activity against *Bacillus cereus* and *Streptococcus aureus*, with the inhibition zone having diameter between 15-18mm. Crude extract EMD showed has activity against all bacteria compare others, an equally good activity against *Escherichia coli*, *Klebsiella pneumonia* and *Streptococcus ubellis*, and has strong activity against *Pseudomonas aeruginosa*, *Bacillus cereus* and *Streptococcus aureus*, with the inhibition zone having diameter between 15mm - 27mm. The lowest activity showed for HMD and CMD (diameter<10mm). Crude extract of MMD and EMD exhibited a strong and moderate radical scavenging with the inhibition percentages are 79% and 76% respectively. Crude extract of HMD and CMD exhibited a low free radical scavenging with the inhibition percentages in the range between 59-69%. EMD and MMD extracts exhibited low mildly cytotoxic effect against HepG2 cell line giving IC50 values between 30-60 µg/mL.

INTRODUCTION: Herbal medicine represents one of the most important fields of traditional medicine all over in the world. Plant is still the main source in searching the new medicine, there is increasing interest in plant as sources of agents to fight microbial diseases. For that, the usage of plants is still the main priority for research because medicine from plants has its own advantages which has low toxicity, easy to get, cheap and has less side effect if it is used in a right dose.

One of this example is *Phaleria macrocarpa* (Scheff) Boerl) from Papua island. Empirically it has been used as a traditional medicine. It is used in two forms. Firstly, in unprocessed form or it can be eaten as it is, like guava. Some people also like to add chillies while eating the fruit salad. It is very dangerous. It has a very serious effect which starts from wounds on the lips and mouth, tongue cannot taste, drunk and even get toxic.

Secondly, it is already processed into ingredients. These ingredients can be combined with other ingredients from other plants ¹.

Phaleria macrocarpa (Scheff.) Boerl. known as Mahkota Dewa has been used traditionally for treatment of cancers in Indonesia and also to cure many diseases like liver, heart, diabetic, skin diseases, rheumatism, anti histamine, and lower the cholesterol level. The therapeutic effect of natural material is directly related to the chemical compound of their host.

Phenolics are the major contributors to the antioxidant activity of fruits, vegetables, grains and many plants ^{2,3}. The antioxidant activity of fruit and seed of *Phaleria macrocarpa* (Scheff.) Boerl. crude extract has been studied ^{4,5,6}.

An excessive phytochemical and biological activities investigation on the *Phaleria macrocarpa* (Scheff) Boerl fruit have been done ^{1,4,7,8}.

Besides that, plant-based antimicrobials have enormous therapeutic potential as they can serve the purpose with lesser side effects to heal many infectious diseases. The *Phaleria macrocarpa* (Scheff.) Boerl. leaves and seed were had anti bacterial activities, but only reported in two bacteria's (*Pseudomonas aeruginosa* and *Bacillus cereus*) ⁹. Because of that, research about antimicrobial of *phaleria macrocarpa* (Scheff.) Boerl. leaves extract still need to investigate with another kinds of bacteria and however, the research on the part of leaves is still limited. Thus, this research conducted to know antibacterial and radical scavenging activities of *Phaleria macrocarpa* (Scheff.) Boerl. leaves and how its cytotoxicity properties in HepG2 cell lines were also need to investigate. Thus, information about *Phaleria Macrocarpa* (Scheff.) Boerl. Leaves became more complete.

MATERIALS AND METHODS:

Chemicals and Reagents: Dimethyl sulphoxide (DMSO), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Iron(III) chloride (FeCl₃), quercetin, were purchased from Sigma(Sigma–Aldrich GmbH, Steinheim, Germany). Nutrient Broth (NB), Mueller Hinton Agar (MH), chloroform, ethyl

acetate, methanol, and n-hexane (Darmstadt, Germany). All other chemicals were analytical grade and obtained from either Sigma or Merck.

***Phaleria macrocarpa* (Scheff) Boerl.) materials:** Sample of *Phaleria Macrocarpa* (Scheff.) Boerl. leaves was collected on July to September 2008 in Bengkulu, Sumatera Island, Indonesia.

Preparations of Crude Extracts and TLC Profiling: The crude extracts were obtained by successive extraction using hexane, chloroform, ethyl acetate and methanol. The dried powder of leaves (500 g) was extracted five times at room temperature for 24 hours. The extract were evaporated under reduced pressure to give 5% hexane (HMD), 6% chloroform (CMD), 2.5% ethyl acetate (EMD), and 3% methanol (MMD). TLC of HMD, CMD and EMD were performed by using TLC plastic sheets (Merck 1.05735.0001) which were pre-coated with silica gel 60F₂₅₄, and MMD on TLC Aluminum sheets (Merck 1.05559.0001) which were pre-coated with RP-18F_{254S}. The TLC visualization was carried out by using UV-254nm, iodine vapour, DPPH TLC bioautographic assay (antioxidant potency tested), reagent spray with vanillin and ferric chloride (FeCl₃).

Antimicrobial Assay:

Bacteria Test: All of crude extracts were tested for antibacterial activity using six bacteria target, namely *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Streptococcus ubellis*, *Streptococcus aureus*, and *Bacillus cereus*. Standard of gentamycin 10 µg (Oxoid, UK) was used in this assay. All the bacterial were obtained from Hospital Sultanah Zahirah Kuala Terengganu.

Agar Well Diffusion Method: All bacteria were cultured in appropriate broths at 30°C for overnight and concentration were adjusted using a spectrophotometer (λ_{max} 600nm) to 10⁵-10⁶ colony forming units (CFU) per mL. Agar cultures were prepared as described by Jorgensen (1987) and bacterial test was performed by the Mueller Hinton agar well diffusion method (modification method of Perez *et al.*, 1990 and Collins *et al.*, 1995).

The suspension was used to inoculate 90 mm diameter Petri dishes with a sterile non-toxic cotton swab on a wooden applicator. The cut agar disks were carefully removed by the use of forceps sterilized by flaming. To each well were introduced different concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.625 mg/mL) of crude extract. Sample of crude extracts (20 μ L) was loaded onto each well.

Then, antibiotic gentamycin was located on the middle of agar surface. Control negative experiment was used solvent (DMSO) with no plant extract. The plates were incubated for 24 hours at 37°C. Clear inhibition zones around the well were measured after the incubation period. All the determinations were performed in triplicates. The clear inhibition zones around the well indicated the presence of antimicrobial activity.

Radical Scavenging Activity Assay: The Radical scavenging activity was measured by the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) method. The ability of extracts to scavenge the α , α -diphenyl- β -picrylhydrazyl (DPPH) radical was measured as reported by Von Gadov *et al.* (1997). 50 μ L of methanolic solution of crude extracts (10 mg/mL) was placed in cuvette and 2 mL of methanolic DPPH (Sigma, Germany) solution (6×10^{-5} M) was added. The decrease of absorbance was determined at 515 nm using BIO RAD Smart Spec Plus Spectrophotometer.

The absorbance measurement commenced immediately and recorded as absorbance at $t = 0$. Then, the test solution was incubated in dark place for 30 minutes before read the absorbance and recorded as absorbance at $t = 30$. The determinations were performed in three replicates. Positive control was used in this method are Quercetin (Sigma, Germany). The radical scavenging activity of the tested sample was expressed as inhibition percentage of the DPPH radical scavenging activity, calculated using this following formula:

$$\% \text{ Inhibition} = [(Abs_{t=0} - Abs_{t=30}) / Abs_{t=0}] \times 100$$

Where, $Abs_{t=0}$ is the absorbance at $t = 0$ and $Abs_{t=30}$ is the absorbance at $t = 30$.

Cytotoxicity Assay:

Cell Culture: Cytotoxicity screening was carried out by using HepG2 cell lines. The HepG2 cell lines were obtained from from the American Type Culture Collection and maintained in plastic culture flasks (Corning Incorporated Corning) as described previously¹⁴. The HepG2 cells were kept in MEM media (Sigma) with 10% fetal bovine serum (ICN Biomedicals, Inc., Aurora, OH) and 1% penicillin-streptomycin at 37°C under 5% CO₂.

MTT Assay: The 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide (MTT) assay, a previously described method was used¹⁴ with some modifications. The MTT assay relies on the production of a colored formazan by the action of mitochondrial enzymes on MTT. For the MTT assay, HepG2 cells were seeded at a density of 2.5×10^5 cells/well on 96-well plastic plates. The test was performed using sterile flat-bottomed 96 well plates. Test samples, EMD and MMD were prepared in varying concentration by serial dilution in MEM medium with concentration of 60, 30, 15, 7.5, 3.75, 1.875, 0.938 and 0.469 μ g/mL. 100 μ L of varying concentration were added into each well and followed by 100 μ L of cells. Then, the plates were incubated for 72 hours at 37°C, 5% CO₂, 90% humidity.

The assay of each concentration was performed in triplicates and untreated well cell population was performed as control wells. After 72 hours, colorimetric MTT method assay was used to measure the living cells. The viability of cells was measured by the amount of blue formazan crystals after 20 μ L fresh MTT solution (5 mg/ mL in PBS) was added to each well. The plate then was incubated for four hours at 37°C, 5% CO₂, 90% humidity.

About 170 μ L of medium was removed from each well. Then 100 μ L of DMSO was added into each well and mix thoroughly by pipettes 10-20 times to dissolved the blue formazan crystals. The plate was left for 30 minutes before reading the absorbance on ELISA reader at 570 nm reference wavelengths. Cytotoxic activity was expressed as fifty-percent Inhibition concentration (IC₅₀), i.e. the concentration that yields 50% inhibition of the treated cells compared to untreated cell control.

Sample which exhibit cytotoxic index $LC_{50} < 30 \mu\text{g/mL}$, were considered to have significant cytotoxic activity

15

RESULTS AND DISCUSSION:

TLC profiling of *Phaleria macrocarpa* leaves Crude

Extracts: The TLC profiling of the crude extracts were done. Based on the result of TLC (**Figure 1**), the best solvent system to isolate the compound of HMD and CMD is hexane: ethyl acetate (7:3, 5:5). Meanwhile the compound of EMD and MMD is chloroform: ethyl acetate (4:6) and water: acetonitril (6:4). Bands with the DPPH scavenging activity were observed as white yellow bands on a purple background, and showing the presence of antioxidant compound. Nevertheless the

intensity white yellow bands displayed by the EMD and MMD TLC profiling were higher than that of HMD and CMD.

This indicated that the antioxidant compound is major in EMD and MMD (with $R_f = 0.65$). Besides that, there were minor DPPH free radical scavenger compound which appeared as yellow band when sprayed with DPPH (Figure 1d) and this band appeared as brown colour on the TLC profiling of the CMD, EMD and MMD extracts when sprayed with 1% FeCl_3 in methanol, indicating the existence of phenolic compound (Figure 1e). This was also proven by the existence of red spot after being sprayed with vanillin-sulfuric acid reagent (Figure 1c).

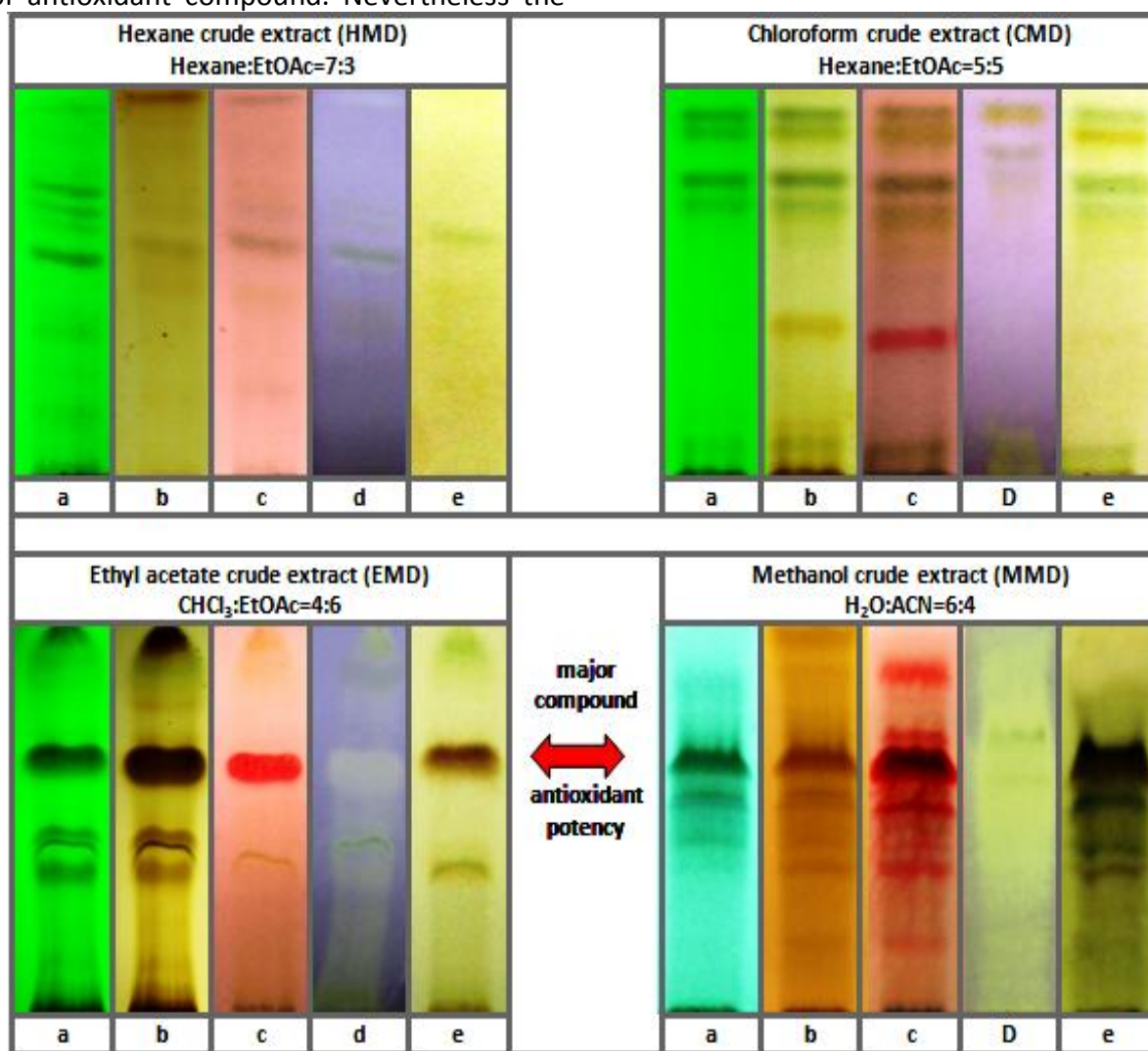


FIG. 1: TLC PROFILING OF *Phaleria macrocarpa* (SCHEFF) BOERL.) LEAVE CRUDE EXTRACTS AFTER VISUALIZATION WITH UV-254nm (A), IODINE VAPOUR (B), VANILLIN-SULFURIC ACID (C), DPPH(D), AND FeCl_3 REAGENT SPRAY (E).

Antibacterial: The EMD showed a strong antibacterial activity against all target bacteria with the inhibition zone of 15-26mm. Similar antibacterial activity was also exhibited by MMD. This crude extract was active towards *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Streptococcus aureus* with inhibition zone of 15-18mm. Meanwhile two other crude extracts, HMD and CMD, were found to have a low antibacterial activity on *Pseudomonas aeruginosa* and *Bacillus cereus* (HMD);

Escherichia coli, *Klebsiella pneumonia*, and *Bacillus cereus* (CMD) with the inhibition zone of < 10mm each (**Table 1**). It was in agreement with the literature range value^{16, 17}. The antibacterial activity of crude extracts of *Phaleria macrocarpa* (Scheff) Boerl leaves were lower compared to antibiotic standard (Gentamycin 10µg), except the antibacterial activity displayed by EMD on *Streptococcus aureus* (diameter 26 mm).

TABLE 1: ANTIBACTERIAL ACTIVITY OF PHALERIA MACROCARPA (SCHEFF) BOERL.) LEAVES CRUDE EXTRACTS

Kind of sample	Code	Bacteria species*					
		<i>E. coli</i>	<i>P.aer</i>	<i>K. pne</i>	<i>B. cer</i>	<i>S. aur</i>	<i>S. ube</i>
Hexane extract	HMD	-	+	-	+	-	+
Chloroform extract	CMD	+	-	+	+	-	-
Ethyl acetate extract	EMD	++	+++	++	+++	+++	++
Methanol extract	MMD	-	++	-	+++	+++	-
Gentamycin standard	GN	22mm	22mm	21mm	25mm	26mm	24mm

* *E. Coli*: *Escherichia coli*, *P.aer*: *Pseudomonas aeruginosa*, *K. pne*: *Klebsiella pneumonia*, *B. cer*: *Bacillus cereus*, *S. aur*: *Streptococcus aureus*, *S.ube*: *Streptococcus ubellis*; *(-) No activity, (+) weak activity (7-10mm halo), (++) good activity (10-15mm halo), (+++) strong activity (≥15mm halo)

Radical Scavenging Ability: Result of above supported by antioxidant activity of four crude extracts of *Phaleria macrocarpa* (Scheff) Boerl.) (Scheff) Boerl.) leaves (**Figure 2**). Crude extract of MMD and EMD exhibited a strong and moderate radical scavenging with the inhibition percentages are 79 and 76% respectively. Crude extract of HMD and CMD exhibited a low free radical scavenging with the inhibition percentages in the range between 59-69%. It was in agreement with the literature range value¹⁷. A strong free scavenging activity with the inhibition percentages in the range of 79-89%, and moderate to weak activity in the range of 55-78% was assessed All extracts have less activity as compared to Quercetin. Quercetin was used as a positive control in this screening.

Phenolics are the major contributors to the antioxidant activity of fruits, vegetables, grains and many plants^{2,3}. There was a correlation between antioxidant activity and total phenolic content of *Phaleria macrocarpa* (Scheff) Boerl.) leave extracts¹⁸. In general, the higher total phenolic content will result in the higher total antioxidant capacity¹⁹. Besides that, other researchers also proved that phenolic compound have a direct relationship with antibacterial effect²⁰.

The free radical DPPH. possesses a characteristic absorption at 517 nm (purple in colour), which decreases significantly on exposure to radical-scavengers (by providing hydrogen atom or electron donation). A lower absorbance at 517 nm indicates a higher radical-scavenging activity of the extract. Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a standard assay in antioxidant activity studies and offers a rapid technique for screening the radical-scavenging ability of specific compounds or extracts²¹.

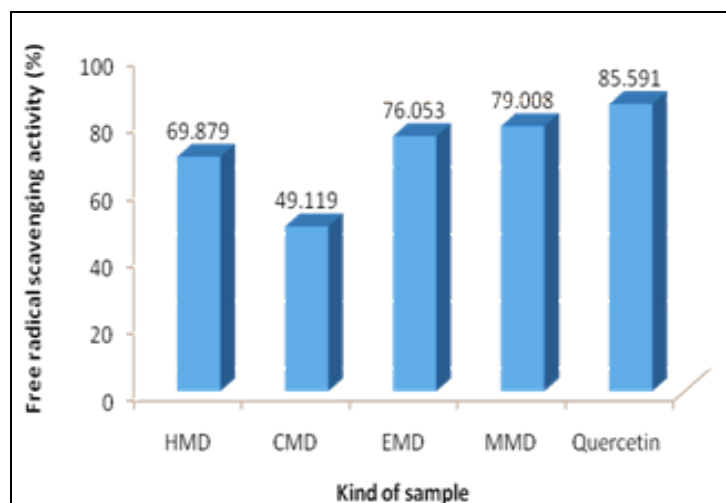


FIG. 2: FREE RADICAL SCAVENGING ACTIVITY OF PHALERIA MACROCARPA (SCHEFF) BOERL.) LEAVES CRUDE EXTRACTS

Cytotoxicity assay: In this research, HepG2 cell lines was used to predict of the toxicity. Human-derived liver cells HepG2 have been extensively used as the test system for the prediction of toxicity, carcinogenicity and cell mutagenecity in humans²². Result in **Figure 3** show that only EMD and MMD were had IC50 value between 30-60 $\mu\text{g}/\text{mL}$ (with IC50 value are 32.5 $\mu\text{g}/\text{mL}$ and 40 $\mu\text{g}/\text{mL}$, respectively), meanwhile the HMD and CMD were had IC50 > 60 $\mu\text{g}/\text{mL}$.

The results were interpreted as follows: IC50 < 1.0 $\mu\text{g}/\text{ml}$ - highly toxic; IC50 1.0-10.0 $\mu\text{g}/\text{ml}$ - toxic; IC50 10.0-30.0 $\mu\text{g}/\text{ml}$ - moderately toxic; IC50 > 30 < 100 $\mu\text{g}/\text{ml}$ - mildly toxic, and > 100 $\mu\text{g}/\text{ml}$ as nontoxic. EMD and MMD crude extracts were considered to have significant mildly toxic activity in HepG2 cell line and had good activity for further investigation process.

However, since reactive oxygen radicals play an important role in carcinogenesis²³. It is therefore possible to suggest that the presence of antioxidants compound in the *Phaleria macrocarpa* (Scheff) Boerl.) leaves extracts may play some role in reducing cell number.

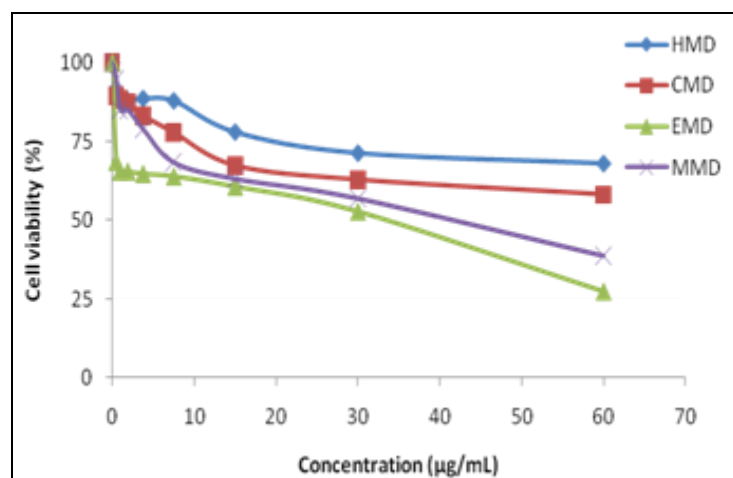


FIG. 3: CYTOTOXICITY PROPERTIES OF EMD AND MMD CRUDE EXTRACT OF *Phaleria macrocarpa* (SCHEFF) BOERL.) LEAVES IN HEPG2 CELL LINES AFTER 72 HOURS

CONCLUSIONS: The study showed that the DPPH free radical scavenging of EMD and MMD should be contributed by phenolic compounds, and showed mildly toxicity in HepG2 cell lines. Presence of antioxidants compound in the *Phaleria macrocarpa* (Scheff) Boerl.) leaves extracts may play some role in

reducing cell number. Furthermore, the natural product derived from *Phaleria macrocarpa* (Scheff) Boerl.) leaves extracts have potential use in the food and/or pharmaceutical industries as antioxidant and antimicrobial agents.

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