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Research Article

Isolation, purification, and identification of catechin from *Rhizophora mucronata* methanol leaf extract (Bakau Kurap)

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Abstract
Rhizophora mucronata (Bakau Kurap) is known as 'Red mangrove' growth in the coastal area of Sabah. The current investigation commenced following the behaviour of proboscis monkeys in Sabah, as they consumed mangrove leaves that were believed to contribute to their high sexual activity. The *R. mucronata* leaf is a natural source of flavonoid. Therefore, the present study aimed to isolate, identify and purify the active compound from *R. mucronata* leaf from Sabah by phytochemical screening and characterization using High-Performance Liquid Chromatography (HPLC), Evaporative Light Scattering Detector-HPLC (ELSD-HPLC) and Chromatography-Mass Spectrometry/Mass Spectrometry (LCMS/MS). Young leaves of *R. mucronata* collected around the coastal area of Sabah were authenticated at the Sabah Forestry Department, Sandakan, Sabah with a voucher specimen, *R.mucronata* (Bakau Kurap); SAN 149220. The leaves were oven-dried at 40 ± 3 °C overnight, powdered and subjected to 50% Soxhlet extraction using methanol: water. The extract was filtered, rotary evaporated and freeze-dried at 4 °C to remove excess solvent. The prepared extract was characterized by its total flavonoid, steroid and saponin contents. From HPLC analysis, the fraction collected matched with the catechin standard and Fraction F4 contained the highest catechin concentration (138.12 ppm). Further purification found that RM-F11 matched the catechin standard at the same retention time (RT) 6.8. Qualitative analysis using LCMS/MS, confirmed that the compound identified was catechin. Meanwhile, in Steroidogenesis, RM-F11 showed potent activity with the value of testosterone hormone released at 230.68 ± 11.2 pg/mL. The result indicated that the components of *R. mucronata* leaf of Sabah had the potential as an aphrodisiac agent.

Keywords: Catechin, Chromatography-Mass Spectrometry/Mass Spectrometry, Flavonoid, High-Performance Liquid Chromatography, *Rhizophora mucronata,* Steroidogenesis

INTRODUCTION

Catechins are flavanols that are polyphenolic substances. Catechin condensation (epicatechin and a catechin epimer) produces condensed or non-hydrolyzable (Sanda *et al.,* 2019). Catechins are categorised as monomer flavan-3-ol compounds. Flavonoids represent the primary bioactive compound found in green tea (Pervin *et al.,* 2019). The levels of Catechins account for 60% to 80% of the total flavonoid content. Catechins' antioxidant properties can prevent low-density lipoprotein oxidation, reduce plasma cholesterol levels, and inhibit platelet aggregation (Ambigaipalan *et al.,* 2020). The C2–C3 double bond at the carbonyl of the C ring and the two hydroxyl groups at positions 3–4 of the B ring play a crucial role in the biological activity of flavonoids (Cardoso *et al.,* 2020). Catechin is known to increase dopamine and norepinephrine levels in rats (Teixeira *et al.,* 2013), which stimulates the dopaminergic pathway and can improve sexual behaviour (Teixeira *et al.,* 2013). According to Al-Snafi (2016), plants serve as helpful sources for various secondary metabolites that are widely used worldwide in various industries, primarily in the manufacture of food technology, pharmaceuticals, and sources of colours and fragrances. Phytochemical screening can be useful in identifying the chemical component of the plant extract and the one that predominates over the others

(Fongang, 2021).

Rhizophora mucronata is a member of the Rhizophoraceae family and is commonly known as loop root mangrove, red mangrove, or Asian mangrove (Grin, 2006). *R. mucronata* is an excellent natural antidiabetic agent due to the presence of phenolics, flavonoids, gallic acid, quercetin, and coumarin (Sur, *et al.,* 2016). Furthermore, the excellent anti-inflammatory activity of the bark extract of *R. mucronata* with the presence of the phytoconstituents lupeol, quercetin, and caffeic acid (Sadeer, *et al.,* 2019). The extracts of *Rhizophora mucronata* made with methanol and ethanol had a greater quantity of beneficial phytochemicals than the extract made with chloroform. Despite this, a higher concentration of terpenoids was discovered in the chloroform extract, which is in line with the results of the earlier investigation (Cowan, 2019). *R. mucronata* leaf contains tannins and flavonoids, which are extra-nutritional components of traditional medicine with the ability to change cell physiology. According to Ghosh *et al.,* (2015), *R. mucronata* leaf contains steroids, triterpenoid, alkaloid, flavonoid, tannin, catechin, quinone, and anthocyanidin. The methanol extract of *R. mucronata* leaf, was documented to be rich in catechin with 47.428 parts per million (ppm).

Despite the medical properties and widespread use in ethnomedicine to treat a wide range of ailments of *R. mucronata* (Salini, 2015), the literature lacks information regarding the plant's medicinal chemistry. Currently, no research has been undertaken to analyse the medicinal components. Khalid *et al.* (2022) conducted a recent study on *R. mucronata* methanol extract, a species of mangrove found in Sabah, which revealed the potential aphrodisiac properties of this plant. The study suggests that the bioactive compound known as catechin is responsible for the elevation of testosterone levels.

Therefore, this research determined the possible active ingredient (catechin) in *R. mucronata* (RM) methanol extract that oversees the activities that increase sexual function as well as the mechanism by which these activities are implanted. The present work aimed to isolate, identify, and quantify flavonoid components present in methanolic extracts of the *R. mucronata* leaves using gradient reversed-phase HPLC with diode array detection and then analysed with HPLC–ESI-MS.

MATERIALS AND METHODS

Chemicals

L-Dopa (Levodopa), estradiol benzoate, and progesterone were all acquired from Sigma Aldrich in the United States. Chemical Dilution Medium (CDM): Dulbecco's Modified Eagle Medium (DMEM) (Thermo Scientific, buffered with sodium bicarbonate) supplemented with 100 IU/mL Penicillin and 100 mg/mL Streptomycin (PAA Lab). Phosphate Buffered Saline (PBS), Calcium and magnesium free (PBS): PBS (Thermo Scientific, buffered with sodium bicarbonate). Culture Trypsin-EDTA (Ethylenediaminetetraacetic acid): 2 mg/mL trypsin (Sigma) and 0.3 mg/mL EDTA in PBS Neutral Red (NR) 8 mg/mL sodium chloride 0.02 mg/mL potassium chloride 0.2 mg/mL potassium dihydrogen orthophosphate 1.15 mg/mL disodium hydrogen orthophosphate Solution Off the Shelf: 2.0 mg/mL NR (Sigma), NR Medium: 50 µg/mL NR in CDM and culture medium, NR 1% glacial acetic acid and 50% ethanol, dimethyl sulfoxide (DMSO), and ethanol are the components of the desorbing solution. Positive control was carried out utilising sodium lauryl sulphate (SLS) (Calbiochem).

Plant materials and preparation of organic extraction

Young leaves of *R. mucronata* (RM) collected around the coastal areas of Sabah were authenticated at the Sabah Forestry Department, Sandakan, Sabah, with a voucher specimen, *Rhizophora mucronata* (Bakau Kurap); SAN 149220. The leaves were washed and oven-dried at 40 ± 3 ºC overnight. The dried leaves were ground to a fine powder using the grinder and successively extracted from non-polar to polar solvent using Soxhlet apparatus for 8 hours (methanol:water ratio at 50%). The extract was filtered and rotary evaporated at 50 ºC for 8 hours to remove excess solvent and 24-hour freeze-dried process. The methanol extract was kept at 4 ºC in the refrigerator until further use. Methanol has been chosen because it is the universal solvent that can dissolve all the active ingredients of secondary metabolites (Mahmiah *et al.,* 2016).

Qualitative analysis of phytochemical screening

The presence of essential phytochemical constituents that typically exhibit biological activities of the RM methanol extract was determined using standard procedures described by Sofowora (1993). These components included alkaloids, saponins, flavonoids, tannins, polyphenolic compounds, triterpenes, and steroids.

Alkaloids

The extract was dissolved in diluted HCl and filtered. The filtrate was mixed with a few drops of Hager's reagent. A prominent yellow precipitate indicates the presence of alkaloid.

Saponins

20 mg of the extract was diluted with distilled water and made up to 10 mL. The suspension was shaken in a graduated cylinder for 15 mins. A prominent layer of foam indicated the presence of saponins.

Flavonoids

The extract was dissolved in distilled water and filtered. The extract was then dissolved and shaken in 10%

ammonia hydroxide solution. The formation of yellow colour in the ammonia layer indicated the presence of flavonoids.

Tannins and polyphenolic

Tannins and polyphenolic compounds were detected by mixing the plant extract with 1% ferric chloride solution. The formation of a blue-black colour indicates the presence of hydrolysable tannins, while brownish-green colour indicates the presence of condensed tannins. A few drops of 10% lead acetate solution were added to the filtrate. A bulky white precipitate indicated the presence of phenol compounds.

Triterpenes and steroids

Triterpenes and steroids were detected using the Liebermann-Buchard reagent. The formation of a reddish colour indicates the presence of triterpenes and a greenish colour for steroids.

Fractionation of *Rhizophora mucronata* (RM) methanol extract using vacuum liquid column

The Vacuum Liquid Column (VLC) was a primitive fractionation technique that initiated the search for single, pure components. Due to its simplicity, VLC has been increasingly utilised in natural products and synthetic chemistry over the past decade (Bucar, 2013). 600 g of RM methanol extract was mixed with as little silica gel (Merck) as possible to produce a dry slurry, which was then loaded onto a VLC column (10 cm in diameter and 13 cm in length) filled with silica gel as stationary phase. The column was eluted stepwise under vacuum with solvents of increasing polarity, ranging from MeOH and water (1:1) as below:

F1: 100% water: 0% Methanol
F2: 75% water: 25% Methanol
F3: 50% water: 50% Methanol
F4: 25% water: 75% Methanol
F5: 0% water: 100% Methanol

After elution with methanol, the column was passed through methanol. The solvents were eluted until the column was empty. Five 500 mL eluant fractions were collected and numbered F1 through F5. The mass of the eluted fractions was determined after evaporating the solvents in a stream of room-temperature air. The collected fractions were evaluated for catechin quantification. The schematic diagram of the extraction and isolation of chemical constituents from leaves of RM is shown in Fig. 1.

RM methanol extract and fraction profiling (Quantification of catechin using HPLC PDA)

Using Shimadzu Liquid Chromatography with Waters X -Bridge reverse phase integrated with SPD-M20A Photo Diode Array detector (PDA), low-pressure gradient pump (LC-20AD), autosampler (7SIL-20AD), and Oven (CTO-20AC), the quantitative analysis of catechin in RM methanol extract and fraction was performed. A C-18 column (4.6 mm x 250 mm, 5 µm) with a thermostat compartment was utilised to isolate compounds. The temperature in the column oven was set to 40 ºC. At a flow rate of 1.0 mL/min for 30 minutes, a gradient solvent system consisting of water (85%): acetonitrile (ACN) (15%) with 0.1% formic acid was utilised. The composition of the initial mobile phase was maintained at 15% solvent A for 10 minutes, changed linearly to 25% and held for 5 minutes, then returned to the initial conditions and held for 10 minutes until the equilibrium of the chromatograph column was reached. 5 mg of Catechin standard (1000 ppm) was dissolved in methanol and topped off to the mark. Additional serial dilutions from stock: 500 ppm, 250 ppm, 125 ppm, 62.5 ppm, and 31.2 ppm. 0.1 g of RM methanol extract and fraction were weighed and dissolved in methanol (10 mg/mL). For analysis, the extract was filtered through a 0.2 µm filter membrane. Injecting 10 µL of filtered RM methanol extract through the HPLC sample injection port. The chromatograms were extracted using a PDA

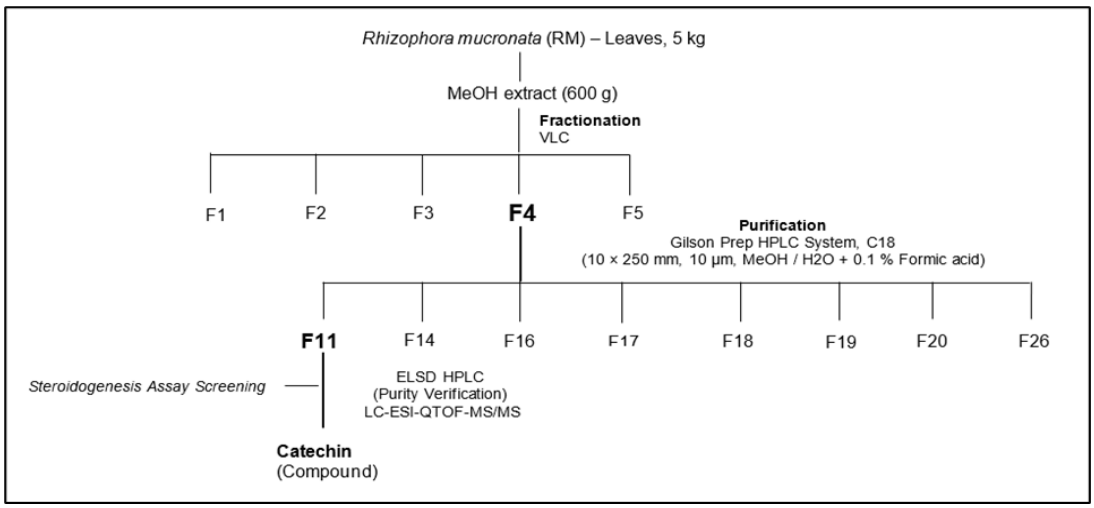


Fig. 1. *Isolation of compound from Rhizophora mucronata leave extract.*

detector at 254 nm. Comparing its UV spectra and retention times enabled the identification of catechin. The quantitative analysis was conducted using a catechin-based calibration curve (y = 1805.13x-+0; R2 = 0.998).

Purification and isolation using preparative HPLC analysis

Preparative High-performance liquid chromatography was conducted to purify all Nano-electrospray ionisation mass spectrometry chemicals. The VLC Fraction with the highest catechin concentration was then purified and isolated. The selected fraction was dissolved in methanol at a concentration of approximately 200 mg/mL, the solution was centrifuged, and 1000 µL was injected into preparative HPLC to isolate the active components. The compound was isolated using a Gilson Preparative HPLC equipped with a GX-281 liquid handler and 254 UV detector. Waters Novapak 25 x 100 mm RPC18 cartridge and a 10 mm guard were utilised in a Prep LC 25 mm column. Chromatographic separation was achieved using an elution gradient from 100% water (0.1% formic acid) to 100% acetonitrile (0.1% formic acid) over 50 minutes at a 12 mL/min flow rate. The wavelengths used for detection were 210 and 254 nm, and eight primary fractions were collected at a rate of 0.5 minutes per fraction. Under vacuum, fractions were evaporated to dryness and then analysed with HPLC-ELSD and LC-ESI-QTOF MS/MS.

Purity verification by HPLC-ELSD

Analytical HPLC-ELSD (Evaporating Light Scattering Detectors) with a standard (Catechin) confirmed the fraction with the highest steroidogenesis concentration and purity. Analysis was conducted on an Agilent 1200 HPLC system (Agilent Corp., USA) comprised of a vacuum degasser, a binary pump, an autosampler, a column compartment, and an ELSD (Alltech Associates, Deerfield, USA). A flow rate of 1.0 mL/min was maintained throughout the analysis. All analyses were conducted at 40 ^{o}C with optimised ELSD conditions including a nitrogen gas flow rate of 1.3 L/min and a drift tube temperature of 45 ^{o}C. The chromatographic separations were performed on a (250 mm x 4.6 mm x 5 µm) YMC ODS-AM column. A and B comprised of 1.0% Formic Acid and Acetonitrile, respectively. Each run was finished in under 24 minutes. The rate of discharge was 1 mL/min. Then, a 20 µL aliquot of fractions and standards was injected for analysis into the HPLC-ELSD system. The wavelength of detection was set to 360 nm.

Isolation of catechin using Liquid Chromatography and Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry (LC-ESI-QTOF-MS/MS)

In the realm of food safety, LC-MS is a common detection method. Due to the high mass accuracy of the TOF mass spectrometer, the combination of LC and ESI-TOF-MS enables the quantitative and qualitative analysis of compounds in complex matrices by reducing matrix interferences. LC has an efficient separation capacity, while MS has a high sensitivity and robust structural characterization capability. The isolation of catechin was performed using an Ultimate 3000 (Dionex). Details on LC-MS operating conditions are provided in Table 1. Column C18 (Hypersil Gold C18 100 x 2.1 mm; 1.9 µm) was utilised to perform the separation at a flow rate of 0.5 mL/min. A 1 µL solution was injected into the column, and separation was performed using gradient elution at 40 ^{o}C with 0.1% Formic Acid in water (A) and purified Acetonitrile (B) as mobile phases. The gradient programme was initiated from 10% to 90% B in 15 minutes, remained at 90% B for 5 minutes, and then returned to 10% B after 10 minutes for a total LC -MS run time of 30 minutes. The system was operated with positive ion polarity, and a full scan analysis from 50 m/z to 1000 m/z was undertaken. 8 L/min of nitrogen was used as the desolvation gas in a dry furnace at 180 °C with the samples.

Steroidogenesis

The H295R (human adenocarcinoma) was obtained from the American Type Culture Collection (ATCC), Manassas, USA (CRL-2128TM). The steroidogenesis assay assesses the effects of RM fractions on testosterone (T) synthesis and gives qualitative data on a fraction's ability to induce or inhibit T production. The cells were put in 15-20 mL of Dulbecco's Modified Eagle's mixture and Ham's F-12 Nutrient Mixture (DMEM/F12) mixture with 2.5% Nu-Serum and 1% ITS + Premix. The medium was replaced 2 –3 times a week. Subcultures were done when cell confluence reached 85–90% every 5-7 days. The experiments used passages 4–13 cells (OECD 456, 2011). As the recommendation (OECD 456, 2011) suggested, all test substances were dissolved in 100% DMSO. Maximum 0.5% DMSO was utilised in wells. Final DMSO concentrations for all fractions were 0.1%. Catechin (4.0 mg/mL) was evaluated in 0.1% and 0.5% solutions and concentrations.

H295R Steroidogenesis assay

Cells were visually inspected for morphology and attachment before the experiment to ensure proper conditions. Microtiter plates were injected with 3 x 10^5 cells per well. Due to its natural steroid levels, particularly Testosterone, Nu-Serum was omitted from the assay's growth media. Seeded plates were incubated at 37 °C in 5% CO_2. After 24 hours, cells were treated to RM fraction (RM-F11), and a solvent control (SC) by adding 1 µL of the solution in triplicate (after replacing the cell medium). The dishes were returned to the incubator at 37 ^{o}C and 5% CO_2. To analyse hormones, 950 µL of each well's cell media was kept at -20 ^{o}C in Eppendorf containers.

Quality control

H295R cells were treated to blank cell media (n = 6), 0.1% DMSO in cell media (SC, n = 6), and positive and negative control on QC plates. Forskolin (FOR) was utilised as a

Table 1. LC-MS Operating Condition

Instrument	Condition
Column	Hypersil Gold RS-tech C18 (100 × 2.1 mm, 1.9 µm)
Oven temperature (ºC)	40
Flow rate (mL/min)	0.5
Injection volume ((µL)	10
Ionization source (positive mode)	ESI
Fragmentor voltage (V)	70
Quadrupole temperature (ºC)	99
Capillary voltage (V)	3000
Nebulizer pressure (psi)	35
Drying gas temperature (ºC)	300
Mass range (m/z) scan mode	200□500
Mobile phase (%)	A: TFA in water B: acetonitrile
Gradient elution composition (%)	(B): 10–25 (0–35 min), 25–10 (35–37 min), 10 (37–45 min)

positive control at doses of 1 and 10 µM, and prochloraz (PROC) as a negative control at concentrations of 0.1 and 1 µM (n = 3 per concentration). To assess cell viability, 950 L of cell medium was removed, 750 µL of fresh medium, and 200 µL of resazurin were added. Plates were incubated for three hours at 37 °C and 5% CO_2. The fluorescence of the produced resorufin was measured at 560 and 580 nm excitation and emission wavelengths using a Tecan Infinite 200 PRO multimode microplate reader.

RESULTS AND DISCUSSION

Preliminary phytochemical properties

Mangroves, particularly *R. mucronata*, produce a wide range of distinctive natural compounds or secondary metabolites with significant pharmacological effects that are used in ethnomedicine to treat a wide range of ailments (Setyawani *et al.,* 2019). Although the chemical, biological, and pharmacological activities of *R. mucronata* leaf extract have not been fully elucidated, it has been established as a natural source of flavonoids (Sadeer, *et al.,* 2019). *R. mucronata* plant components, including bark, collar, hypocotyl, and stilt root, have been intensively examined for its phytoconstituents with significant medicinal activity, and its structure revealed that *R. mucronata* composed of benzophenone and ethanol (Joel, *et al.,* 2021). Research by Jairaman *et al.* (2020), on *R. mucronata* bark extract, the chemicals squalene (19.19%), hexadecanoic acid (6.59%), and oleic acid (2.88%) were identified in this plant. However, no information is available regarding the qualitative phytochemical screening and characterization of the *R. mucronata* methanol leaf extract of Sabah mangrove species.

The relative concentration of the secondary metabolites in the samples is shown in Table 2 and was calculated by considering the colour intensity of the test-positive results. Flavonoids, saponins, and steroids in the *R. mucronata* methanol extract were found to be moderate. Additionally, a small amount of tannin was found. The *R. mucronata* leaf extract was free of any alkaloids. This outcome was consistent with Rohini *et al*. (1999) findings that *R. mucronata* leaf extract is a naturally occurring source of tannins and flavonoids, primarily catechin. It has also been proven that *R. mucronata* methanolic extract is a catechin-rich mangrove (Suganthy and Devi, 2016).

Identification of catechin through Analytical HPLC Peak Profiling (HPLC PDA)

HPLC-PDA method effectively detects and measures catechin from RM methanol extracts and the peak component's uniqueness demonstrated the peak's purity. The HPLC quantification method was validated, and the results are shown in Fig. 2 and Fig. 3, respectively. The calibration curve showed excellent linearity with a correlation coefficient of 0.9983 or higher.

The HPLC identification process revealed that both the RM methanol extract and VLC fractions exhibited identical retention time as the standard solution. It was demonstrated that the methanol extract of RM and its VLC fractions contain catechin. Based on the chromatogram in Fig. 4, the RM methanol extract (M50) was found to have a concentration of catechin at 72.01 ppm. Meanwhile, the findings from the study on RM VLC M50 fractions indicate significant variations in the utilisation of various percentage concentrations. Among the different fractions, the one with a composition of 75% methanol and 25% water (F4) contains the highest amount of catechin, measuring 138.12 ppm (Fig. 5). In comparison, the other RM VLC M50 fractions (Fig. 6 - F1 (0% methanol: 100% water), F2 (25% methanol: 75% water), F3 (50% methanol: 50% water), and F5 (100% methanol: 0% water) - have catechin concentrations of 0.00 ppm, 16.07 ppm, 108.3 ppm, and 57.9 ppm, respectively. Therefore, F4 was selected for further isolation and purification using Preparative HPLC.

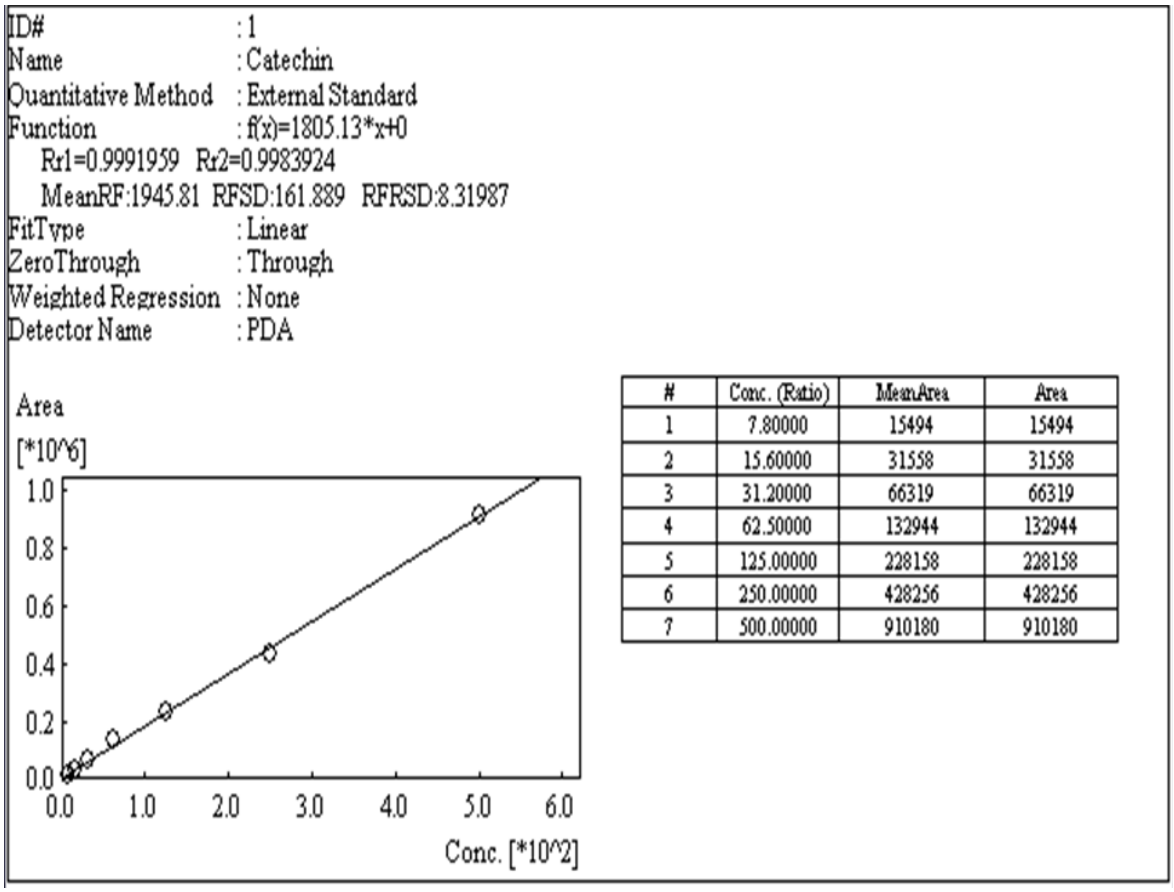
Prep HPLC analysis

Purified compounds are required for compound identification and structural elucidation in synthesis or natural product chemistry. F4 fraction (150 mg/mL) was dissolved in methanol:water (50:50) and subjected to reversed-phase C18 preparative HPLC. From the preparative chromatographic separation, five prominent fractions, namely RM-F9, RM-F10, RM-F11, RM-F14 and RM-F16 were collected in test tubes and dried (Fig. 7). The fractions' weight (mg) ranged from 4 mg to 20 mg from 150 mg of Fraction F4 (Table 3). All fractions isolated and collected by Prep HPLC, were then further ana-

Table 2. Phytochemical screening of RM methanol extract

Alkaloids	Saponins	Flavonoids	Tannins & Polyphenolic compounds	Triterpenes & Steroids
-	++	++	+	++

* Absent (-), low concentration (+), moderate concentration (++), high concentration (+++)



#	Conc. (Ratio)	MeanArea	Area
1	7.80000	15494	15494
2	15.60000	31558	31558
3	31.20000	66319	66319
4	62.50000	132944	132944
5	125.00000	228158	228158
6	250.00000	428256	428256
7	500.00000	910180	910180

Fig. 2. *Calibration curve of catechin standard*

lysed with Catechin standard using HPLC ELSD to verify its purity.

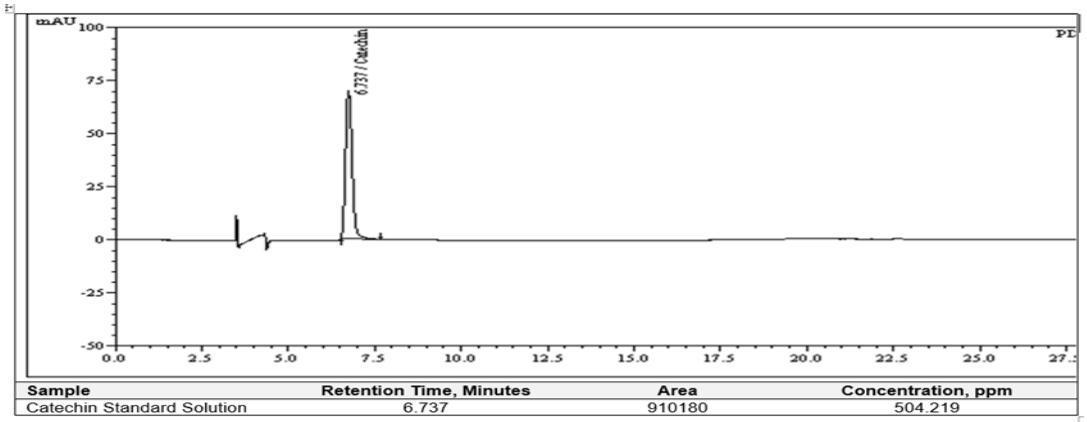
HPLC-ELSD (Evaporative Light Scattering Detector) analysis

This test used a reference standard and an HPLC-ELSD detector to verify whether the intended peak had been purely isolated. In HPLC analysis, ELSD is often used. The ELSD can identify samples with lower volatility than the mobile phase and is not affected by the optical characteristics of the drug under test or the functional groups of the substance. Furthermore, the ELSD is temperature insensitive; it has baseline stability suitable for gradient elution of the liquid phase combination; and is more receptive to separating of these compounds.

The ELSD general detection strategy can resolve the major difficulties that plagued prior detection approaches. HPLC-ELSD is a useful approach for evaluating the content of catechins in diverse medicinal plants (Igelige *et al.,* 2014). Other studies that used HPLC coupled with ultraviolet and a mass spectrometer (HPLC-UV-MS), HPLC coupled with an evaporative light scattering detection (HPLC-ELSD), and HPLC-UV-ESI-MS methods to analyse catechin provided additional support for this profile identification (You *et al.,* 2012). From the identification performed via HPLC, it is safe to conclude that the peak of interest in RM methanol extract is catechin since it has the same retention time as standard catechin. Among all fractions collected, RM-F11

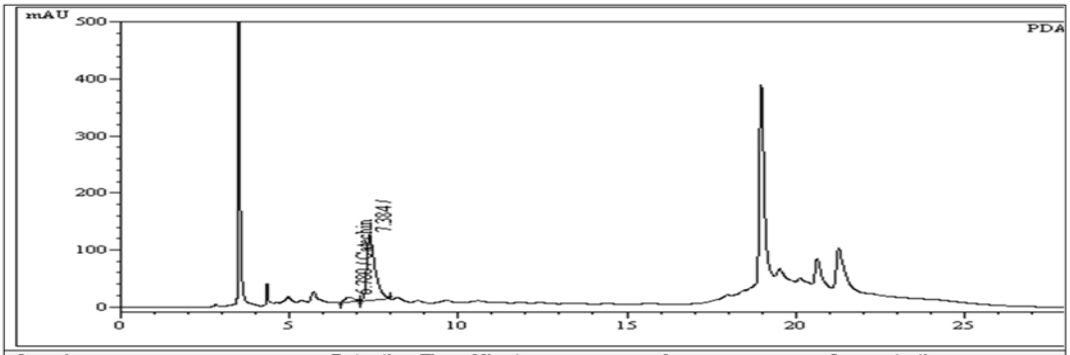
Table 3. Five prominent RM fractions and the weight collected via Prep HPLC

Fraction	Weight (mg)
RM-F9	20.0
RM-10	11.0
RM-F11	12.0
RM-F14	20.0
RM-F16	4.0



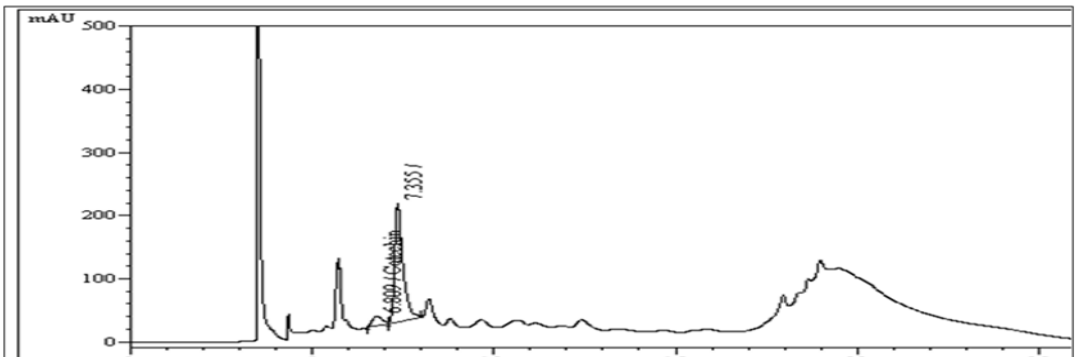
Sample	Retention Time, Minutes	Area	Concentration, ppm
Catechin Standard Solution	6.737	910180	504.219

Fig. 3. *Catechin standard solution*



Sample	Retention Time, Minutes	Area	Concentration, ppm
RM methanol extract (M50)	6.780	147487	72.01

Fig. 4. *Catechin quantification in RM methanol extract (M50)*



Sample	Retention Time, Minutes	Area	Concentration, ppm
Fraction F4 of VLC M50	6.809	282883	138.12

Fig. 5. *Catechin quantification in RM fraction F4 of VLC M50*

matched the Catechin standard at the same retention time (RT) RT 6.8 (Fig. 8). Fraction RM-F11 underwent analysis using LC-ESI-QTOF/MS for compound identification and characterization. Meanwhile, the steroidogenesis assay was conducted in this study to validate of its ability to release hormone (T).

Compound identification using Liquid Chromatography-ESI-QTOF/MS

The LC-ESI-QTOF/MS method has proven useful for detecting and characterising chemicals in various plants (Gu, 2019). Profiling using LC-ESI-QTOF-MS gives important information for understanding the effects on flavonoid component profiles. The retention time (RT), a mass discrepancy between observed and theoretical mass (10 ppm), and mass spectrometric (MS) data acquired under positive electron spray ionisation mode (ESI) were used to identify and characterise substances.

Ionising the molecules allowed the mass spectrophotometer to separate and determine the mass-to-charge ratio. The source of ions generated and the duration of analysis for separating the produced ions are two major factors in this procedure. The bioactive

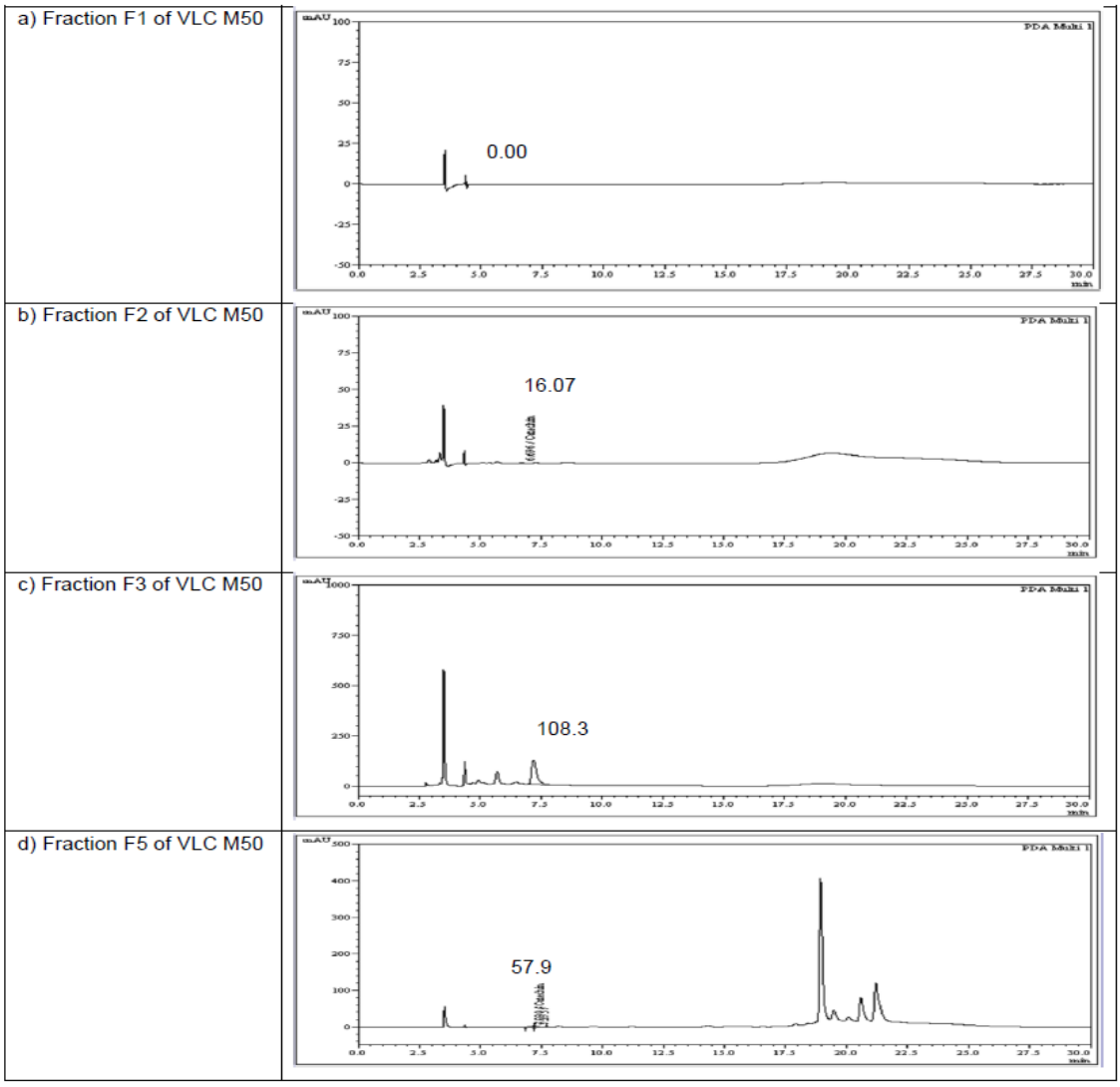


Fig. 6. *Catechin quantification in RM VLC fraction a) Fraction F1 of VLC M50, b) Fraction F2 of VLC M50, c) Fraction F3 of VLC M50 and d) Fraction F5of VLC M50*

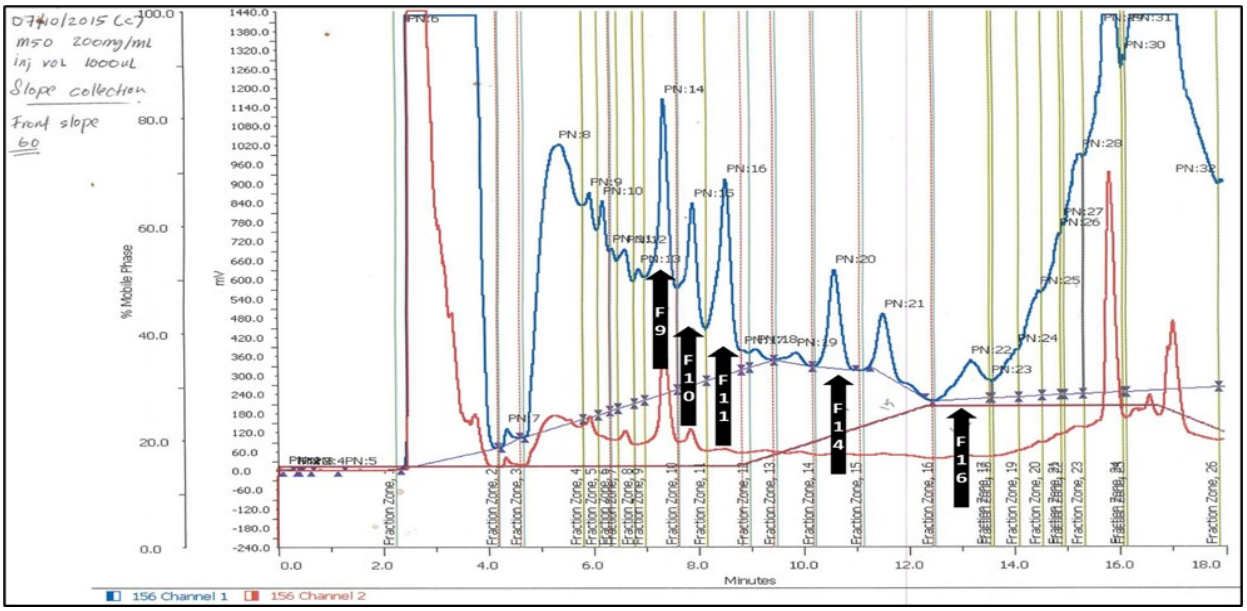


Fig. 7. *Gilson Preparative HPLC C18: Water Novapak Column; Condition: gradient of 100% water (0.1% Formic Acid) to 100% Acetonitrile (0.1% Formic Acid) over a duration of 50 mins, with a flow rate of 12 mL/min.*

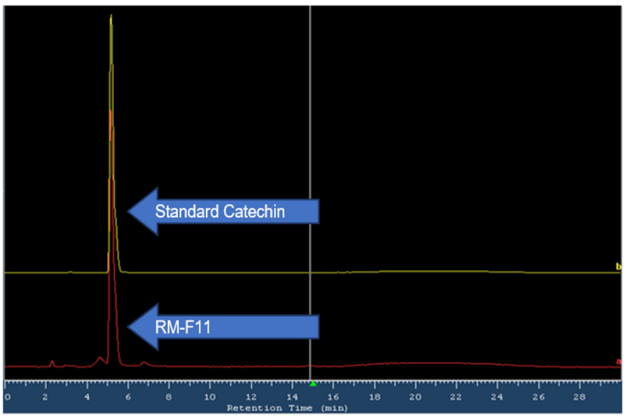


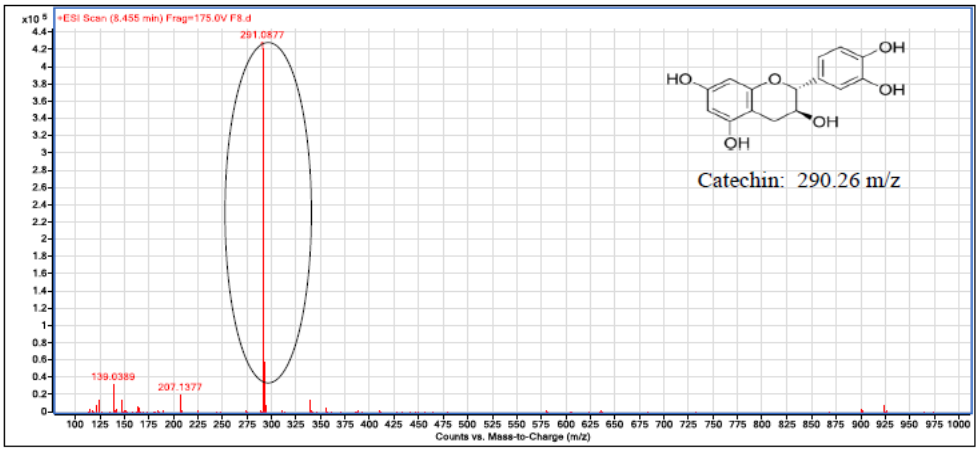
Fig. 8. *RM-F11 overlay with catechin standard*

components in this extract were identified using the ESI-MS technique. Meanwhile, the quadrupole MS validates the target compounds further. This approach was utilised in another investigation to identify Catechins. Mandal *et al.,* (2022) discovered 24 metabolites, 10 synthesised by LC-ESI-QTOF/MS, including Catechin, in green tea extract employing a positive ionisation mode.

The presence of the compound was confirmed based on the comparison of m/z values from the MS2 spectra with the literature. A positive ionization mode was used to identify catechin. Identification of peaks of catechin isolates in RM-F11 showed RT 6.8 min; $[M+H]^+$ at *m/z*, 291.26 (Fig. 9). The compound's molecular weight detected was 291.26 and matched the targeted molecular weight of catechin which is 290.26. Since positive mode was performed, the molecular weight reported needs to be minus one to get the actual molecular weight of the compound detected. Therefore, the results obtained from HPLC peak identification and LC-ESI-QTOF/MS data confirmed that the fraction collected (RT: 6.8 min) is a Catechin compound. The LC-ESI-QTOF/MS method was successfully tuned for the qualitative analysis of RM-F11 and completely separated catechin.

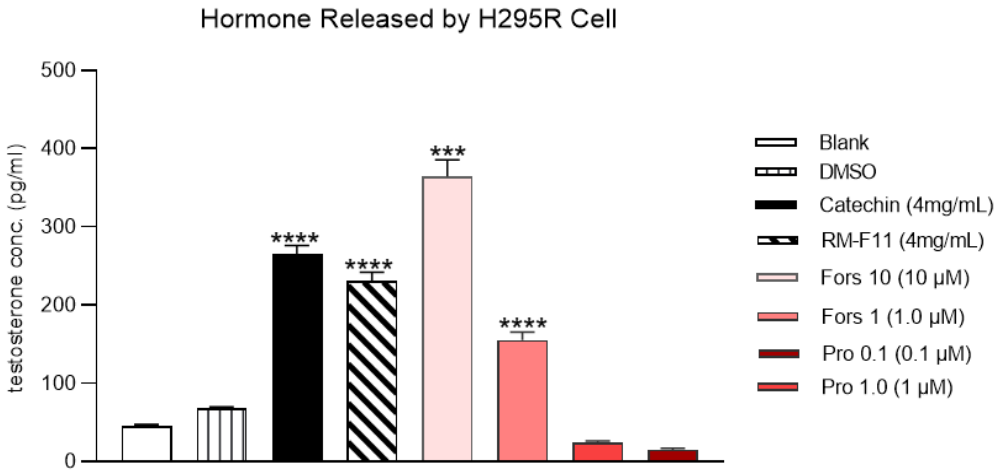
Steroidogenesis

Testosterone was produced and concentrated *in vitro* using a process according to OECD 456 criteria to evaluate whether RM methanol extract promotes or inhibits endocrine receptor function. The need to assess endocrine disruption caused by methods other than receptor-mediated mechanisms, and the impacts of endocrine-disrupting substances, led to the development of an H295R steroidogenesis assay (Hecker *et al.,* 2007). The *in vitro* approach has been demonstrated to be useful in determining the ability of individual chemicals, medicines, pesticides, and their mixes to modify steroidogenesis (Xu *et al*., 2006). H295R cells exhibit the full steroidogenesis pathway, essential for mineralocorticoids, glucocorticoids, and steroid sex hormone production. Furthermore, the cells can be used to investigate steroidogenic pathways at multiple levels of organisation, such as gene expression enzyme abundance and activity (Sanderson *et al.,* 2002), and the hormone products of these catalytic enzyme activities (Hecker *et al.,* 2007). As a result, H295R cells provide an ideal *in vitro* model for investigating the impacts and mechanisms of endocrine-disrupting drugs' interactions with steroidogenesis. Standard Catechin (commercial agent) showed potent activity with the highest value of hormone released at 265.67 ± 10.4 pg/mL, not significant compared to RM-F11. RM-F11 showed strong activity in inducing testosterone with a hor-



Compound	Molecular Formula	Retention Time (RT) (min)	Molecular Weight	Theoretical (m/z)	Observed (m/z)
Catechin	$C_{15}H_{14}O_6$	6.8	290.26	290.26	291.26

Fig. 9. *LC-ESI-QTOF/MS (positive mode) mass spectra of HPLC peak fraction (RT: 6.8)*



Group	Blank	DMSO	Catechin	RM-F11	Fors 10	Fors 1	Pro 1	Pro 0.1
Testosterone Concentration (pg/mL)	45.82 ±1.3	67.69 ±2.2	265.67 ±10.4 ***	230.68 ±11.2 ***	364.44 ±21.2 ***	155.01 ±10.5 ****	24.01 ±2.3	14.92 ±1.9

Fig. 10. *Effect of Catechin (commercial agent) and sample fractions (RM-F11) on hormone release (Testosterone) in H295R cells. Values represent the mean ± S.D with P>0.05 (comparison with DMSO). All samples (Catechin, RM-F11) at tested concentration 4 mg/mL. Forskolin (Fors) at concentration 10 µM and 1 µM were used as positive control, while Prochloraz (Pro) at concentration 1 µM and 0.1 µM were used as negative control*

mone level of 230.68 ± 11.2 pg/mL, significant (****p<0.0001) than the vehicle control (DMSO)(Fig.10). These findings demonstrated that RM methanol extract does not act as a hormone disruptor within the given parameters. The results of the steroidogenesis assays showed that RM methanol extract stimulates testosterone expression in Human Adrenocortical Carcinoma cell lines. The steroidogenic effects of this group of flavonoids have been documented (Yu *et al*., 2010). Identifying many flavonoids that may improve StAR gene expression and testosterone production via various processes is feasible. According to Padashetty and Mishra (2007), flavonoids in plant extract may play a part in changing testosterone levels. It may also be the cause of the increased male sexual behaviour observed in this study. Animal studies on flavonoid-enhanced testosterone synthesis and reproductive function show the possibility of using natural flavonoids to prevent the reduction in testosterone. According to Yu *et al*. (2010), catechin enhanced plasma testosterone in male rats *in vivo*. In an *in vitro* investigation using low-dose concentrations of catechin, the release of testosterone hormone by the Leydig Cells of male rats was stimulated by human chorionic gonadotropin (hCG).

Conclusion

RM methanol extract (M50) analysis showed a catechin concentration of 72.01 ppm. Meanwhile, the study's findings revealed that fraction F4 contained the highest catechin concentration, measuring 138.12 ppm. Fraction F4 exhibited the highest levels of testosterone. Through preparative chromatographic separation of fraction F4, five distinct fractions were obtained. Among these, fraction RM-F11 was found to have the same retention time (RT) of 6.8 as the Catechin standard. The compound identification of the isolated peaks in RM-F11 was confirmed through LC-ESI-QTOF/MS analysis, which revealed a molecular weight of 291.26 for catechin. Fraction RM-F11 exhibited significant potency of producing testosterone release in the Steroidogenesis assay, comparable to that of catechin.

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Conflict of interest

The authors declare that they have no conflict of interest.

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