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Genotypic identification and catechin profiling of *Uncaria gambir* in West Sumatra, Indonesia

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Abstract. *Wardi ES, Syukur S, Chaidir Z, Jamsari J. 2024. Genotypic identification and catechin profiling of* Uncaria gambir *in West Sumatra, Indonesia. Biodiversitas 25: 1151-1158.* The primary objective of this study is to assess genetic and chemical variability among four genotypes of *Uncaria gambir* cultivated in West Sumatra. *Uncaria gambir* is native to Indonesia, widely cultivated in West Sumatra, used as an herbal remedy, and become a significant agricultural product for Indonesian export with catechin content as a standard. The phytochemical components of this species have been extensively studied, and its extracts are employed in traditional medicine. To fingerprint genomic DNA, we employed three pairs of Specific Sequence-Related Amplified Polymorphism (SRAP) markers selected from 29 combinations tested. A total of 150 amplified bands were generated, highlighting substantial genetic variability among the tested genotypes. Notably, the highest amplified and polymorphic band was obtained with primer E combination from three selected primers (primer E, primer F, and primer T). In addition, only catechins were identified among the four types of catechins employed as HPLC standards. We detected varying catechin concentrations among the genotypes, with genotype Mancik exhibiting the highest concentration. This finding underscores the potential for genotype-specific bioactive marker identification within *U. gambir*. In addition, this study shows the efficiency of SRAP markers in genotype identification within *U. gambir*. Furthermore, the identification of catechin as a prominent bioactive marker emphasizes its significance for further exploration and conservation efforts.

Keywords: Biomarker, catechin, DNA fingerprint, *Uncaria gambir*

INTRODUCTION

Gambir (*Uncaria gambir* (W.Hunter) Roxb.) is a medicinal plant and a highly sought-after export commodity in Indonesia (Ferita et al. 2013). Belonging to the Rubiaceae family, Gambir is also cultivated in Malaysia and various other Southeast Asian regions (Munggari et al. 2022). This plant grows in West Sumatra, North Sumatra, Riau, and South Sumatra in Indonesia (Yunarto et al. 2023). Traditionally, Gambir has been used for the treatment of a wide range of ailments, including gastrointestinal diseases (Syukur et al. 2022), wounds, stomachaches (Fahmi et al. 2023), diarrhea, and diabetes (Ferita et al. 2013).

Uncaria gambir plants can reach heights of around 2.4 m and have leaves of 8 to 14 cm long and 4 to 6.5 cm broad. Each pair of leaves may have a pair of globular inflorescences, and yellowish flowers can be developed at the base of the leaves. The tubular, hairy blooms are carried in 6 to 8-centimeter globose heads. The fruits are less than 2 cm long and nearly cylindrical. *Uncaria gambir* plants can only be grown in a variety of soil types with a pH range of 4.8 to 5.5 under particular circumstances. The plant needs to be grown at altitudes between 200 and 800 meters above sea level, with high annual rainfall, high humidity, and a 15% slope to the land (Saad et al. 2020).

Gambier, derived from *U. gambir* extract, has evolved into a substantial agricultural commodity for Indonesia's export market, earning global acclaim, and experiencing consistent growth in worldwide production, with approximately 80% originating from West Sumatra province (Widiyarti et al. 2020) with 2,491.39 tons of production in 2018 (BPS 2020). On the local front, gambier holds cultural significance and is widely consumed in various traditional preparations.

Recent studies have thoroughly explored the phytochemical composition of Gambir and its potential therapeutic effects. Gambir contains alkaloids and flavonoids, with catechin identified as the primary compound (Das 1974; Ferdinal 2014). Subsequent research has unveiled several distinct catechin types within Gambir, such as epicatechin, gallocatechin, epigallocatechin, and epicatechin gallate (Auliana et al. 2022). Furthermore, Gambir contains other notable compounds, including gambiriin, hyperoside, isoquercitrin, and pyrocatechol (Taniguchi et al. 2007). It is worth noting that the catechin content serves as a key quality standard for Gambir in Indonesia, as defined by the Indonesian National Standard (Badan Standar Nasional 2000).

In the West Sumatra region, which stands as the largest producer of Gambir in Indonesia, four distinct genotypes of Gambir are cultivated: Udang, Riau, Mancik, and Cubadak

(Ferita et al. 2013). Prior studies have primarily focused on morphological identification to assess genotypic diversity. In our previous research, we initiated molecular identification through DNA barcoding, utilizing ITS (Wardi et al. 2022), rbcL (Wardi et al. 2023), and matK (Wardi et al. 2021) barcodes. While these methods successfully differentiated Gambir genotypes, they proved time-consuming and costly due to the sequencing process.

Furthermore, various molecular marker techniques are available, including RAPD, RFLP, AFLP, iPBS, ISSR, SSR, and SRAP. Among these, SRAP is a recognized, simple, efficient, and cost-effective marker system applicable to various molecular biology studies like genetic diversity analysis, genomic and cDNA fingerprinting, map construction, gene tagging, and map-based cloning. SRAP is distinctive for targeting Open Reading Frame (ORF) sequences in the genome, providing rich genetic information linked to observable traits. Successful application of SRAP hinges on carefully selecting optimal forward and reverse primer pairs for effective polymorphism characterization (Robarts and Wolfe 2014).

In this study, our primary objective is to determine Gambir's genetic variability by employing SRAP markers, a cost-effective, one-step, and highly repeatable marker system (Robarts and Wolfe 2014). Additionally, we aim to investigate catechin variability among the four genotypes, shedding light on the diversity within this valuable plant species. This research holds significant implications for understanding Gambir's genetic diversity and the catechin content variation among different genotypes. The SRAP markers offer a practical and efficient approach to achieve this goal, with potential applications in enhancing Gambir cultivation and maximizing its medicinal properties.

MATERIALS AND METHODS

Plant materials

Fresh leaves from four genotypic varieties of Gambir, namely Riau (R), Mancik (M), Udang (U), and Cubadak (C), were harvested in Siguntur Village, Pesisir Selatan District, West Sumatra Province, Indonesia (Figure 1). These genotypes were selected due to their unique characteristics that are relevant to our study, which aims to explore genetic variations and catechin content.

Plant preparation

Upon harvesting, the leaves were divided into two equal portions in the laboratory. One-half of each sample was subjected to microwave treatment (Panasonic, 800 W for 2 minutes) to terminate all polyphenol oxidase activities and then stored at -80°C until catechin extraction according to Liu et al. (2015). This microwave step serves the purpose of preserving the samples, ensuring that their integrity is maintained for subsequent analysis. The other half was used for DNA extraction.

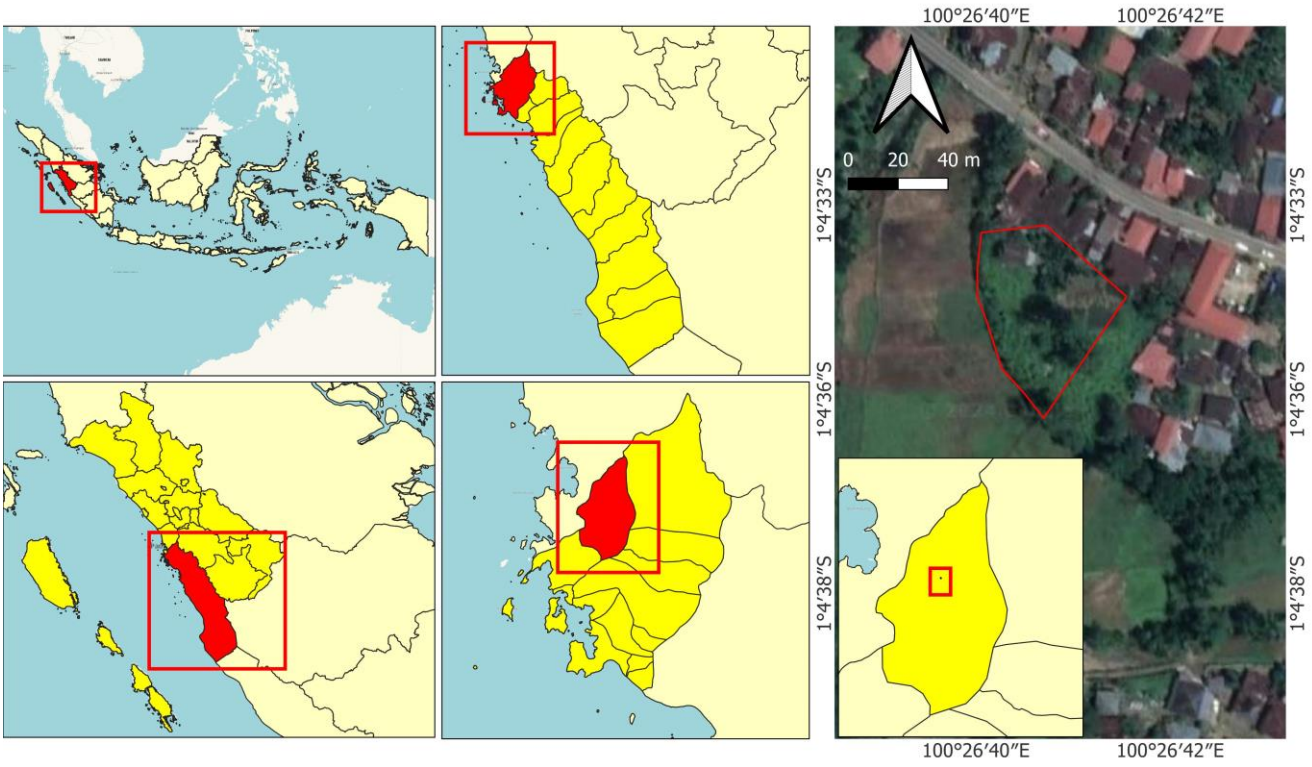

Figure 1. Location of Siguntur village, Pesisir Selatan District, West Sumatra Province, Indonesia indicating the sampling sites of *Uncaria gambir*: Red mark (1°04'34" S, 100°26'40" E)

Table 1. List sequences of primer used in this study

Forward Primer		Reverse Primer	
Name	**Sequence**	**Name**	**Sequence**
Me1	TGAGTCCAAACCGGATA	Em3	GACTGCGTACGAATTGAC
Me2	TGAGTCCAAACCGGAGC	Em4	GACTGCGTACGAATTTGA
Me3	TGAGTCCAAACCGGAAT	Em6	GACTGCGTACGAATTGCA
Me4	TGAGTCCAAACCGGACC	Em8	GACTGCGTACGAATTCAC
Me6	TGAGTCCAAACCGGACA	Em9	GACTGCGTACGAATTCAG
Me8	TGAGTCCAAACCGGACT	Em10	GACTGCGTACGAATTCAT
Me9	TGAGTCCAAACCGGAGG	Em11	GACTGCGTACGAATTCTA
Me10	TGAGTCCAAACCGGAAA	Em12	GACTGCGTACGAATTCTC
Me11	TGAGTCCAAACCGGAAC	Em13	GACTGCGTACGAATTCTG
Me12	TGAGTCCAAACCGGAGA	Em14	GACTGCGTACGAATTCTT
Me13	TGAGTCCAAACCGGAAG	Em16	GACTGCGTACGAATTGTC

Table 2. Sequence-Related Amplified Polymorphism (SRAP) analysis of *U. gambir*

Number	Forward	Reverse	Name
1	Me1	Em6	A
2		Em12	B
3		Em16	C
4	Me2	Em4	D
5		Em12	E
6		Em14	F
7		Em16	G
8	Me3	Em3	H
9		Em9	I
10		Em11	J
11		Em12	K
12		Em14	L
13	Me4	Em11	M
14		Em14	N
15		Em16	O
16	Me6	Em3	P
17	Me9	Em10	Q
18		Em11	T
19		Em14	U
20	Me10	Em13	V
21		Em16	W
22	Me11	Em10	X
23		Em11	Y
24		Em12	Z
25	Me12	Em9	AB
26		Em11	BC
27		Em16	CD
28	Me13	Em8	DE
29		Em9	EF

Primer Selection and SRAP Analysis

For the genetic analysis, we employed a set of 11 forward and 11 reverse primers (Table 1), resulting in 29 possible combinations (Table 2). Each SRAP primer combination was scored as 1 for the presence or 0 for the absence of bands in each sample. Subsequently, we utilized NTSys to calculate the percentage of polymorphic loci, construct a genetic distance matrix, and generate a dendrogram (Rohlf 2015).

DNA extraction

Genomic DNA (25 ng/µL) was extracted from the preserved samples using the CTAB method. The frozen leaf sample (300 g) was crushed and placed in a 2 mL Eppendorf tube. Then, 1 mL of 2x CTAB buffer along with mercaptoethanol was introduced and mixed until uniform. The mixture was then kept at 65°C for half an hour, with occasional inversion every 10 minutes. Following this, 500 µL of phenol:chloroform:isoamylalcohol solution (25:24:1) was incorporated, mixed for a minute, and then centrifuged at 12,000 rpm for 10 minutes. The resulting supernatant was carefully transferred to a new 2 mL Eppendorf tube. 500 µL of chloroform mixture:isoamylalcohol (24:1) was added, mixed for 10 minutes, and then centrifuged at 12,000 rpm for 10 minutes. The supernatant was moved to a new 1.5 mL Eppendorf tube. Sodium acetate was added in an amount equal to 1/10 of the supernatant's volume, followed by the addition of 1 mL of 99% cold ethanol. This was swirled for a minute and then centrifuged at 12,000 rpm for 5 minutes. The resulting supernatant was removed, and 500 µL of 70% ethanol was added. The solution was centrifuged again at 12,000 rpm for 5 minutes and the supernatant was removed. Finally, 100 µL of 1x TE buffer was added to the dried DNA, which was then stored at -20°C. PCR amplification followed a modified protocol based on Li and Quiros (2001). The reaction mixture consisted of genomic DNA (3 µL), KOD One TM PCR Master Mix-Blue (TOYOBO, 13 µL), SRAP primer (2 µL, 10 mM), and nuclease-free water (7 µL), resulting in a total reaction volume of 25 µL.

PCR amplification

The amplification process was started with initial denaturation at 94°C for 5 minutes. Denaturation at 94°C for 1 minute (5 cycles). Annealing at 35°C for 1 minute. Elongation at 72°C for 1 minute (35 cycles). Annealing at 50°C, followed by a final elongation at 72°C for 8 minutes. The amplification results were visualized using electrophoresis with a 1.5% (w/v) agarose gel.

Catechin extraction and HPLC

Catechin was extracted from 0.2 g of leaf material following a modified HPLC procedure (Tao et al. 2016). The leaves were mashed, placed in a 10 mL centrifuge tube, and extracted with 6 mL of 70% methanol (v/v). The tube was heated at 80°C for 20 minutes in a water bath (Sugold) and then centrifuged (Thermo Scientific SL 16 R) for 15 minutes at 4°C and 10,000 rpm. The resulting

supernatant was filtered through a 0.45 µm organic membrane, and methanol was added to dilute the supernatant to 100 mL.

For quantification, we used Catechin (C), Gallocatechin (GC), Epicatechin Gallate (ECG), and Epigallocatechin gallate (EGCG) standards purchased from Merck. A standard curve was prepared by diluting the standards in 70% methanol (v/v). High-Performance Liquid Chromatography (HPLC) was conducted with an injection volume of 10 µL, utilizing a C18 250 x 4.6 mm column at 30°C. The mobile phase consisted of acetonitrile (Mobile phase A) and formic acid 0.2% (v/v) (Mobile phase B). The flow rate was set at 1 mL/min, and detection occurred at a wavelength of 276 nm using a DAD detector.

RESULTS AND DISCUSSIONS

PCR amplification

All 29 SRAP primer combinations produced polymorphic fragments (Figure 2). Subsequently, we selected three primer pairs that generated the highest percentage of clear polymorphic fragments for further analysis. This selection was based on Mishra et al. (2011), which investigated genetic variations in the *Coffea arabica* hybrid. The results demonstrated the effectiveness of the SRAP technique in identifying cultivars and analyzing hybrid coffee species (Rubiaceae family).

Genetic diversity

To assess the genetic diversity of Gambir Udang, Gambir Cubadak, Gambir Mancik, and Gambir Riau, we used these three selected SRAP primers: E, F, and T. This analysis resulted in the generation of 150 amplified bands (Figure 3) with polymorphic band percentages ranging from 35% to 55% (Table 3). Notably, the primer combination 'E' yielded the highest number of amplified and polymorphic bands. However, it is important to mention that primer 'E' and 'F' demonstrated comparable values of Polymorphism Information Content (PIC). PIC is a measure used to assess the informativeness of a genetic marker in revealing polymorphisms within a population.

Table 3. Genetic diversity of *Uncaria gambir*

	PIC	Amplified band	Polymorphic bands	Band size (bp)	% Polymorphic band
Primer E	0.33024	18	10	150-12000	55.56%
Primer F	0.33163	14	5	500-12000	35.71%
Primer T	0.14795	14	6	80-2750	42.86%

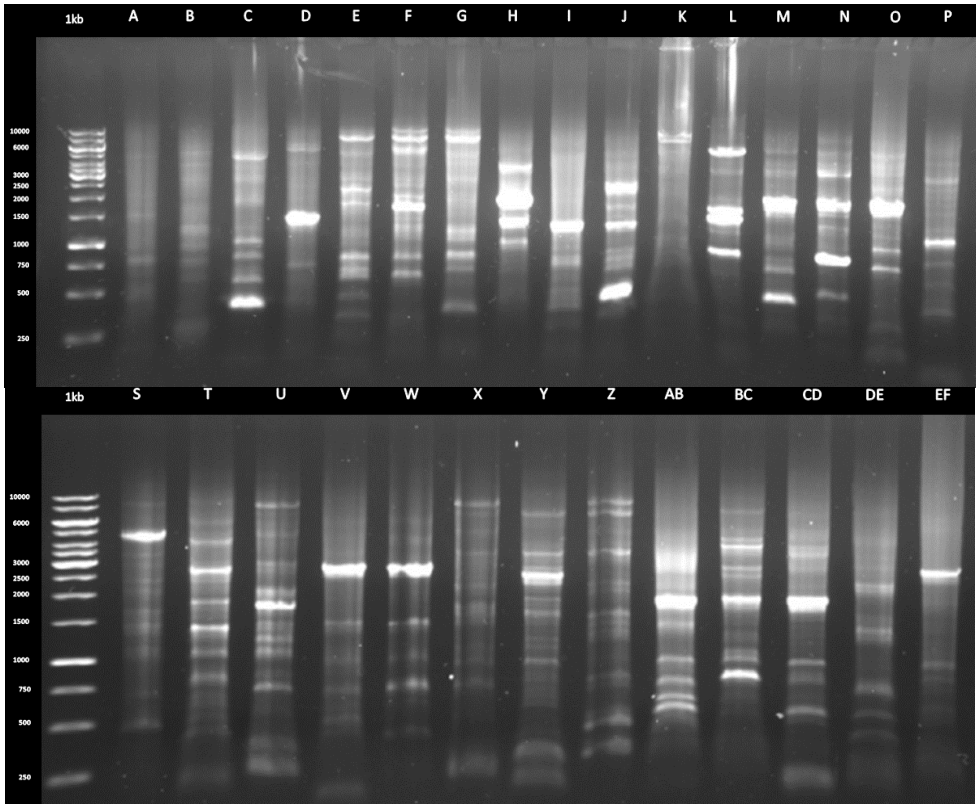

Figure 2. SRAP Analysis of DNA Fragments. 1 Kb: DNA ladder; Each subsequent lane (A-EF) corresponds to a different SRAP primer combination according to Table 2

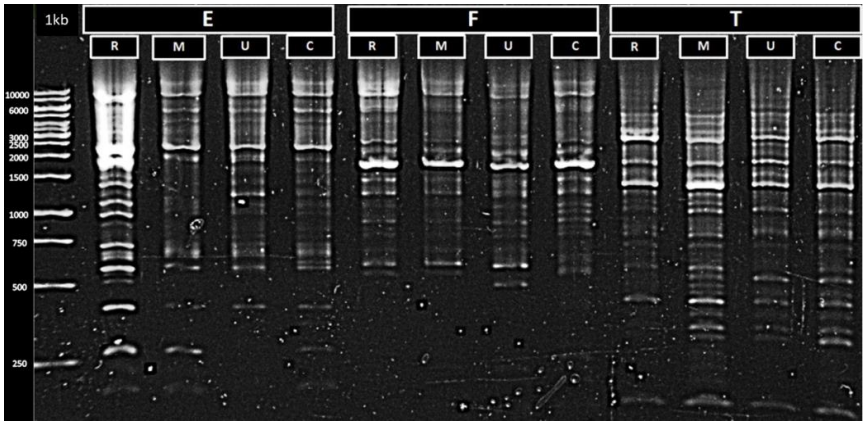

Figure 3. Amplification of SRAP primer to 4 genotypic of *U. gambir*. 1kb: DNA ladder; E, F, T: SRAP primer combination; R: *U. gambir* Riau; M: *U. gambir* Mancik; U: *U. gambir* Udang; C: *U. gambir* Cubadak

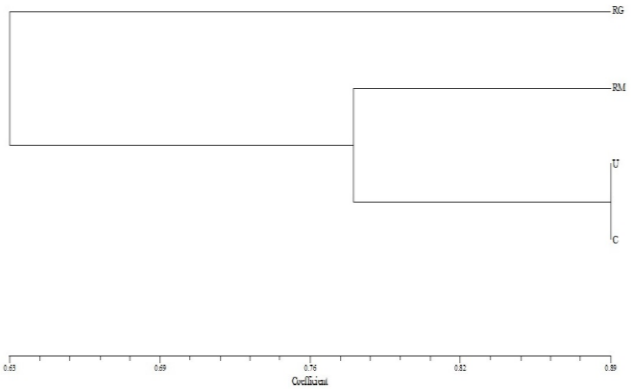

Figure 4. Dendrogram (NTSYS) representing phylogenetic relationship between *Uncaria gambir* based on SRAP marker genetic similarity matrix data using primer combination E. RG: *U. gambir* Riau; RM: *U. gambir* Mancik; U: *U. gambir* Udang; C: *U.* Cubadak

Table 4. Genetic similarity index of four genotypes of *U. gambir* using primer E combination

	RG	RM	U	C
RG	1			
RM	0.5555556	1		
U	0.6111111	0.7222222	1	
C	0.7222222	0.8333333	0.8888889	1

Furthermore, the similarity matrix reveals that primer 'E' exhibited the lowest similarity index among the Gambir varieties, while primer 'F' displayed the highest similarity index (Table 4). Phylogenetic tree analysis grouped Gambir Riau into a distinct clade, while Gambir Udang clustered in the same group as Gambir Mancik and Gambir Cubadak (Figure 4). DNA molecular markers are crucial in phylogenetic studies because of their user-friendly nature, and depending on the chosen type; they can potentially encompass a large portion of the genome. SRAP markers are evenly spread throughout the genome, although their amplification is somewhat reduced near the centromere and telomere. Nevertheless, SRAPs offer several benefits compared to other methods, as they are straightforward, efficient, dependable, and more cost-effective (Cheng et al. 2016). It is important to note that our previous research, which employed DNA barcodes ITS (ITS-u4 and ITS-u1), also segregated Gambir Riau from the others in a similar clade (Wardi et al. 2022). Conversely, DNA barcode rbcL only provided amplification results for Gambir Cubadak and Gambir Riau, preventing comprehensive conclusions from being drawn from that dataset (Wardi et al. 2023).

Significance of SRAP markers in genomic analysis

Recognizing and evaluating genetic diversity and phylogenetic relationships in plants requires appropriate markers. Traditional methods based on morphological features are often ambiguous due to environmental influences (Wrigley et al. 1987; Aneja et al. 2013). However, the choice of markers for genomic analysis is crucial. The SRAP marker system, characterized by its simplicity and effectiveness, has proven valuable in various plant studies. As SRAP markers are PCR-based, they require minimal genomic DNA and exhibit high levels of polymorphism across a wide range of plant species. Importantly, these markers have the capability to be utilized without any prior comprehension of the genome sequence (Aneja et al. 2013). Since first introduced in 2001, SRAP has evolved into a novel and practical PCR marker approach for cultivar identification, gene cloning, molecular mapping, and germplasm characterization in plants (Li and Quiros 2001).

Numerous research endeavors have aimed to explore the use of SRAP for assessing the diversity of various *Uncaria* species. SRAP successfully illustrated genetic diversity in both individual populations and across eight distinct natural populations of *U. guianensis* (Honório et al. 2018). SRAP markers proved highly effective in identifying *U. tomentosa* genotypes. SRAP analysis outcomes indicated that genetic diversity within *U. tomentosa* populations exceeded that between populations. Moreover, the genetic makeup of these populations adhered to an island model (Honório et al. 2017).

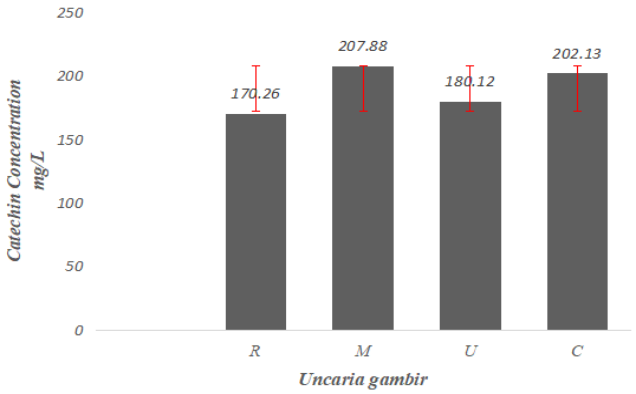

Figure 5. Mean concentrations of *Uncaria gambir* catechin. R: *Uncaria gambir* Riau, M: *Uncaria gambir* Mancik, U: *Uncaria gambir* Udang, C: *Uncaria gambir* Cubadak

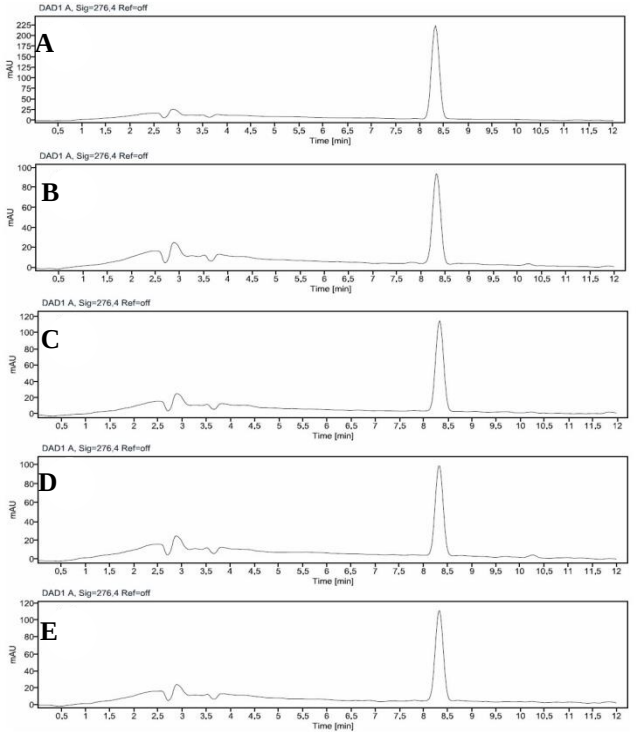

Figure 6. HPLC analysis of catechin. A. Catechin standard, B. *Uncaria gambir* Riau, C. *Uncaria gambir* Mancik, D. *Uncaria gambir* Udang, E. *Uncaria gambir* Cubadak

SRAP markers were initially designed for gene tagging in *Brassica oleracea* L (Li and Quiros 2001). Their purpose was to selectively amplify coding regions of the genome, targeting GC-rich exons with forward primers and AT-rich promoters, introns, and spacers with reverse primers. These primers are approximately 17 or 18 nucleotides in length, with core sequences spanning 13 to 14 bases. The first 10 or 11 bases at the 5' end serve as "filler" sequences and do not possess any specific structure. They are followed by either the sequence CCGG- (for forward primers) or -AATT (for reverse primers). After this core sequence, three random selective nucleotides are included at the 3' end. While various PCR amplification techniques, especially annealing temperatures, have been adjusted to suit specific research requirements, the original protocol was outlined as a two-phase process. This process starts with an initial 4-minute denaturation at 94°C, followed by a template generation phase comprising five cycles of 1-minute denaturation at 94°C, low-temperature annealing at around 35°C, and elongation steps at 72°C. This template is then amplified over 35 similar cycles using a higher annealing temperature of approximately 50°C, and concludes with an extended elongation step of 2 minutes at 72°C. The most common method for scoring fragments has been through a basic presence/absence (0,1) assessment using conventional electrophoresis and gel visualization. Although this method has likely been chosen to reduce costs, it is worth noting that forward primers can be fluorescently labeled, and amplicons can be scored through capillary electrophoresis (Zagorcheva et al. 2020).

Several comparative studies have revealed that SRAP markers offer similar levels of variation as AFLP markers, but with considerably less technical complexity and cost for achieving comparable band-pattern variability and reproducibility. Additionally, up to 20% of examined SRAP markers have shown codominance, a higher rate compared to what has been previously reported for AFLP markers. These aspects underscore the significance of SRAP markers in investigating non-model systems that haven't been explored before, and in experimental projects, particularly in developing countries. However, as SRAP markers are relatively recent and their usage is still in its early stages, any limitations have not yet been thoroughly described (Yi et al. 2021).

Variation of catechin in *Uncaria gambir*

Concentrations of catechin varied among the four varieties of Gambir (Figure 5), measured in milligrams per Liter. Gambir Mancik exhibited the highest catechin content, registering a concentration of 207.88 mg/L, while gambir Cubadak, gambir Udang, and gambir Riau showed concentrations of 202.13 mg/L, 180.12 mg/L, and 170.26 mg/L, respectively. Specifically, only catechin was detected in all samples from these varieties (Figure 6). Research study conducted by Taniguchi et al. (2007) has demonstrated that catechin and epicatechin can be produced from methanol, ethanol, and ethyl acetate extracts. Furthermore, aside from catechin, gambir is also comprised of gallocatechin, epigallocatechin, and epicatechin gallate, as outlined in an alternate investigation that employed the RP-HPLC technique to retrieve these substances from ethyl acetate extractions (Kassim et al. 2011).

Catechins are polyphenolic compounds that present in numerous medicinal plants, primarily sourced from *Camellia sinensis.* They are tannins of the condensation type, possessing a ring and the fundamental structure of flavan-3-ol. Catechins exhibit various chemical structural attributes, including hydroxyl groups (−OH), which readily bind with other substances (Bae et al. 2020). Extracting catechins presents challenges for two main reasons First, they can be bound with sugars or proteins in plant tissue or form

polymerized derivatives with varying solubility. Additionally, their chemical structures and interactions with other food components are not completely understood. Second, catechins are highly prone to oxidation, especially in the presence of light, high temperatures, and alkaline environments, making the development of an effective extraction process quite complex. During extraction, solvents penetrate the solid plant material and dissolve compounds with similar polarity. Common extraction techniques encompass maceration, infusion, heat flux solvent extraction (Soxhlet), microwave-assisted extraction, ultrasound extraction (sonication), and supercritical carbon dioxide extraction. Variables like pH, temperature, solvent-to-material ratio, and the number and duration of individual extraction steps significantly influence the extraction process (Gadkari and Balaraman 2015).

Plants produce catechin through the flavonoid pathway. The initial enzyme in this pathway, Chalcone Synthase (CHS), triggers the reaction that forms chalcone. Chalcone Isomerase (CHI) then facilitates the conversion of chalcone into flavanones. Following this, Flavanone 3-Hydroxylase (F3H), Flavonoid 3′-Hydroxylase (F3′H), Flavonoid 3′5′-Hydroxylase (F3′5′H), and Dihydroflavonol 4-Reductase (DFR) work together to reduce flavanones into leucoanthocyanidin. At this juncture, the pathway splits: on one branch, Leucoanthocyanidin 4-Reductase (LAR) transforms leucoanthocyanidin into GC, and C; whereas partial leucoanthocyanidins are converted into EGC and EC through the sequential action of anthocyanidin synthase (ANS) and Anthocyanidin Reductase (ANR) (Hong et al. 2014).

Catechin content plays a significant role in gambir, as catechins exhibit various pharmacological effects. The high concentration of phenolic compounds in gambir contributes to its antioxidant activities, with catechins being the most prevalent phenolic components utilized for antioxidant purposes. The amount of catechin content strongly correlates with antioxidant activity, suggesting that gambir holds potential as an anticancer agent. Previous studies on gambir have explored the anti-cancer effects of gambir on in vitro T47D breast cancer cells (Syarifah et al. 2019). These findings have illustrated that gambir exhibited a reduction in the proliferation of breast cancer cells, albeit with a lesser potency compared to the positive control medication, Doxorubicin (DOX). Other studies evaluated the inhibitory activity of gambir extract and (+)-catechins against various microorganisms. Microdilution assays were employed for antibacterial testing, revealing that catechins exhibited appreciable activity compared to gambir extract (Musdja et al. 2018). Catechin also displayed exceptional bioactivity in preventing the growth of the wood-rotting fungus *S. commune* (Nandika et al. 2019). Furthermore, catechins in gambir have been connected to various biological functions. These functions include regulating hyperuricemia (Spanou et al. 2012), hindering helminthic parasites (Nitave and Annasaheb 2014), preventing lipid peroxidation (Ningsih et al. 2014), diminishing inflammation (Yimam et al. 2015), handling hyperlipidemia (Yunarto et al. 2021), and managing hyperglycemia (Widiyarti et al. 2012; Hidayati and Rahmatulloh 2022).

In conclusion, SRAP markers are efficient at identifying the genotypes of *U. gambir* with primer E showed high polymorphism power compared to the other two selected primers. Therefore, it can be concluded that urgent conservation efforts are needed for *U. gambir* to prevent the loss of valuable genetic diversity. The creation of germplasm banks is essential for guaranteeing its continued existence. Furthermore, since catechin is the bioactive marker of *U. gambir*, and the pharmaceutical industry has a high demand for high-quality specimens of this medicinal plant, we recommend giving priority to the conservation and management of catechin-rich populations.

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