

# Identification of Epicatechin Gallate and Other Phytochemicals in Methanol Extract of Fresh and Dried Star-Fruits (*Averrhoa carambola* Linn.) for Treatment of Type 2 Diabetes Mellitus

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**Abstract.** From an *in-silico* study, *Averrhoa carambola* L. (star fruit) contains epicatechin gallate (ECG), which has a similar effect to *Sodium-glucose co transporter 2 (SGLT2) inhibitor*. This study aimed to identify ECG and other phytochemicals in methanol extracts of fresh and dried star fruits. Ripened star-fruits (RSFs) were divided into two parts: directly extracted using methanol, while the other was dried and were then extracted using methanol. ECG and phytochemicals were identified using GC-MS, LC-MS and spectrophotometer. The yield of fresh RSFs extract was approximately double than that of dried RSFs extract. 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl- was the highest phytochemicals in fresh RSFs extract whereas dried RSFs extract had glycerine. However, extract of dried RSFs had higher ECG levels than that of fresh RSFs ( $p=0.002$ ). In conclusion, methanol extract of dried RSFs has higher ECG levels and different phytochemicals than fresh RSFs, which potentially become a diabetes herbal drug for an SGLT2 inhibitor.

**Keywords:** Sodium-Glucose Co-Transporter 2 inhibitor, Star Fruits Extract, Epicatechin Gallate, Type 2 Diabetes Mellitus

## 1 Introduction

The prevalence of type 2 diabetes mellitus (T2DM) has been rising in this decade, which increases the number from 4.7% in 1980 to 8.5% in 2014 [1]. Three in four people with diabetes are between the ages of 20 and 64 years old [2]. Therefore, younger adults with diabetes mellitus need oral hypoglycemic drugs to improve insulin secretion, reduce insulin resistance, and increase glucose uptake to lower blood glucose levels [3].

*Sodium-glucose co-transporter 2 inhibitor* (SGLT2i) is a glucose-lowering drug that inhibiting glucose and sodium reabsorption in the kidneys, thus resulting in glucosuria (calorie loss). Consequently, the effect includes reductions in blood glucose levels and decreases in body weight and adiposity, potentially addressing the caloric excess [4–6]. Dapagliflozin is the SGLT2 inhibitor has the most clinical data to date, and other SGLT2 inhibitor are actively being

developed [7]. However, long-term use of this medicine might cause genital infections, ketoacidosis and dehydration [8,9]. As a result of these adverse effects, we are exploring for natural substances that work similarly to SGLT2i.

SGLT2 was validated with dapagliflozin with a binding affinity of 9.0 kcal/mol. The standard interacted with SGLT2 at the residues Asn<sup>75</sup>, Gly<sup>79</sup> and His<sup>80</sup>. From 422 samples, 29 had lower binding affinities than dapagliflozin, but only ECG had similar binding interaction with dapagliflozin at all three residues. ECG in *Averrhoa carambola* was predicted to be a more potent SGLT2i than dapagliflozin based on computational analysis [10].

*Averrhoa carambola* L. (sweet star fruit) has high primary and secondary metabolites such as polyphenols that can modulate the gut microbiota [11]. From an *in silico* study, *Averrhoa carambola* L. (star fruit) contains a secondary metabolite (ECG) that can interact with the SGLT2 protein, as same dapagliflozin as at Asn<sup>75</sup>, Gly<sup>79</sup>, and His<sup>80</sup> [10]. Methanol was the best solvent for the extraction of ECG [12]. Therefore, we used star fruit extracted with methanol. So far, there has not been reported the content of ECG in star fruit. Thus this study aimed to identify ECG and other phytochemicals in methanol extracts of fresh and dried star fruits.

## 2 Research Methods

### 2.1 Extraction of Star Fruits

Ripened star-fruits (RSFs) in this study were purchased from a farmer at Demak, Central Java. Before extracted, RSFs were washed carefully with tap water and divided randomly into fresh and dry parts. Both parts were chopped into small slices ( $\pm 0.5$  cm), but only the dry part was oven-dried at 40°C for 36 hours. Then fresh and dry RSFs were made simplicia by using a disintegrator for 3 min. Simplicia of fresh RSFs was extracted using an existing method with some modifications [12–14]. A total amount of fresh RSFs Simplicia was mixed with chilled methanol for 5 min with a ratio of 1:2. After centrifugation, the suspension was filtered using 0.45  $\mu$ m membrane paper, and the supernatant was dried using a vacuum evaporator at 45°C for 4 hours and a blower evaporator for 24 hours. Meanwhile, Simplicia of dry RSFs was extracted using the maceration method with methanol solvent. This method was adopted from Kallithraka *et al.* (1995) with slight modification. One part of Simplicia was dissolved in three regions of methanol solvent [12]. The dissolved solution was filtered using 0.45  $\mu$ m membrane paper to get the supernatant. To obtain a solid extract, the supernatant was dried as same as the fresh RSFs extract.

### 2.2 Identification of ECG Compound

The standard ECG compound was obtained from Sigma-Aldrich USA. For ECG analysis using GC-MS, fresh and dry RSFs extracts were diluted in methanol to reach 100 ppm final concentration. One  $\mu$ l diluted sample was injected into a ZB-5MS (30 m x 0.25 mm x 0.25  $\mu$ m) column (Phenomenex®, Torrance, CA, USA), linked to a Trace IQS LT mass detector (San José, CA, USA) (GC-MS) and a Thermo Scientific AI 1310 automatic injector (San José, CA, USA) at 300 °C. A flow rate of chemical analysis was set up at 1 ml/min. The spectrum of chemical compounds in the samples was identified using the standard chemical compounds in the NIST library.

Another method used to identify ECG compounds in the fresh and dry RSFs extracts was the LC-MS with a C18 reverse-phase BioSuite™ column (4.6 x 150 mm, Waters Corporation). The mobile phase was made up of (A) 0.5 % formic acid in water and (B) acetonitrile was used to separate ECG from other chemical compounds, and a 20 µL sample was injected into the column with 0.6 mL/min flow rate. The following was the concentration gradient of mobile phase: 0–5 min, 90:10 (A:B); 5–7 min, 90:10 to 86:14 (linear gradient); 7–17 min at 86:14; 17–19 min, 86:14 to 75:25 (linear gradient); 19–24 min at 75:25; 24–25 min, 75:25 to 10:90 (linear gradient); 25–31 min held at 10:90; 31–32 min, 10:90 to 90:10; 32–40 min at 90:10 [15]. Data of peak and retention time of fresh and dry RSFs extracts were compared with the Waters Masslynx 4.1 library data.

### 2.3 Quantification of ECG Compound

A UV-visible spectrophotometer was used to determine ECG compounds in the fresh and dry RSFs extracts compared to the diluted standard ECG from 0 to 20 mg/L. The standard ECG and RSFs extracts were diluted with methanol to make various concentrations. Three different samples of fresh and dry RSFs quotes were measured with a spectrophotometer at 277 nm. A regression linear was drawn to calculate ECG concentration in the new and dry RSFs extracts.

### 2.4 Statistical Analysis

Data of ECG concentrations in fresh and dry RSFs extracts were provided as mean ± standard deviation (S.D.) and statistically analyzed with the independent t-test. A statistical difference used *P*-value <0.05.

## 3 Results and Discussion

### 3.1 Fresh RSFs Yielded More Extract than Dried RSFs

**Table 1.** Rendement of star fruit extracts.

	RSFs (g)	Simplicia (g)	Solvent Ratio	Time (min)	Extract (g)	Yield (%)
Fresh	44.44	-	1 g: 2 ml	20	12.70	28.58
Dried	6,000.00	2,357.77	1 g: 3.33 ml	3	801.64	13.36

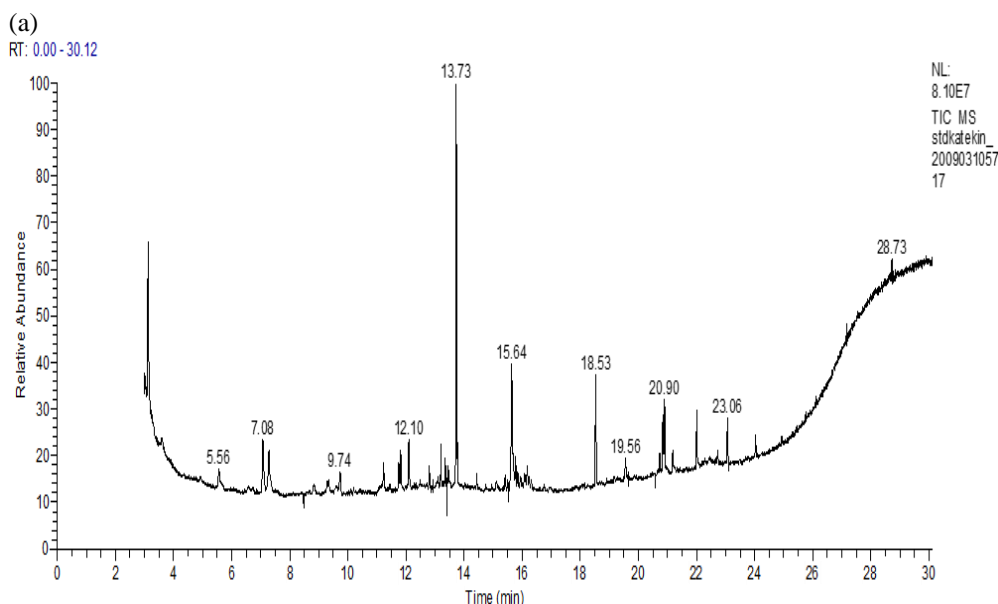
Table 1 summarized the extract yield of fresh and dried RSFs. Fresh RSFs yielded 2.14 times higher extracts than dried RSFs because high temperatures may lead polymers (e.g., carbohydrates and proteins) to degrade and labile compounds (e.g., glucosinolates or lipids) to oxidize [16]. During the drying process, the proportion of phenolic compounds in dried RSFs also reduced because it could be attributed to the degradation of heat-sensitive phenolic compounds [13].

The research finding in our study is different from a previous study in that freeze-dried RSFs with chloroform-methanol combination yielded a 7% fraction [17]. Pothasak *et al.* (2020) found that RSFs extraction using a freeze-drying technique produced 5.33% extract, lower than the yield of our RSFs section [18]. Another study also reported that extraction using an air-dried method yielded 14.86% extracts, but Chinese researchers used star fruit leaves [19].

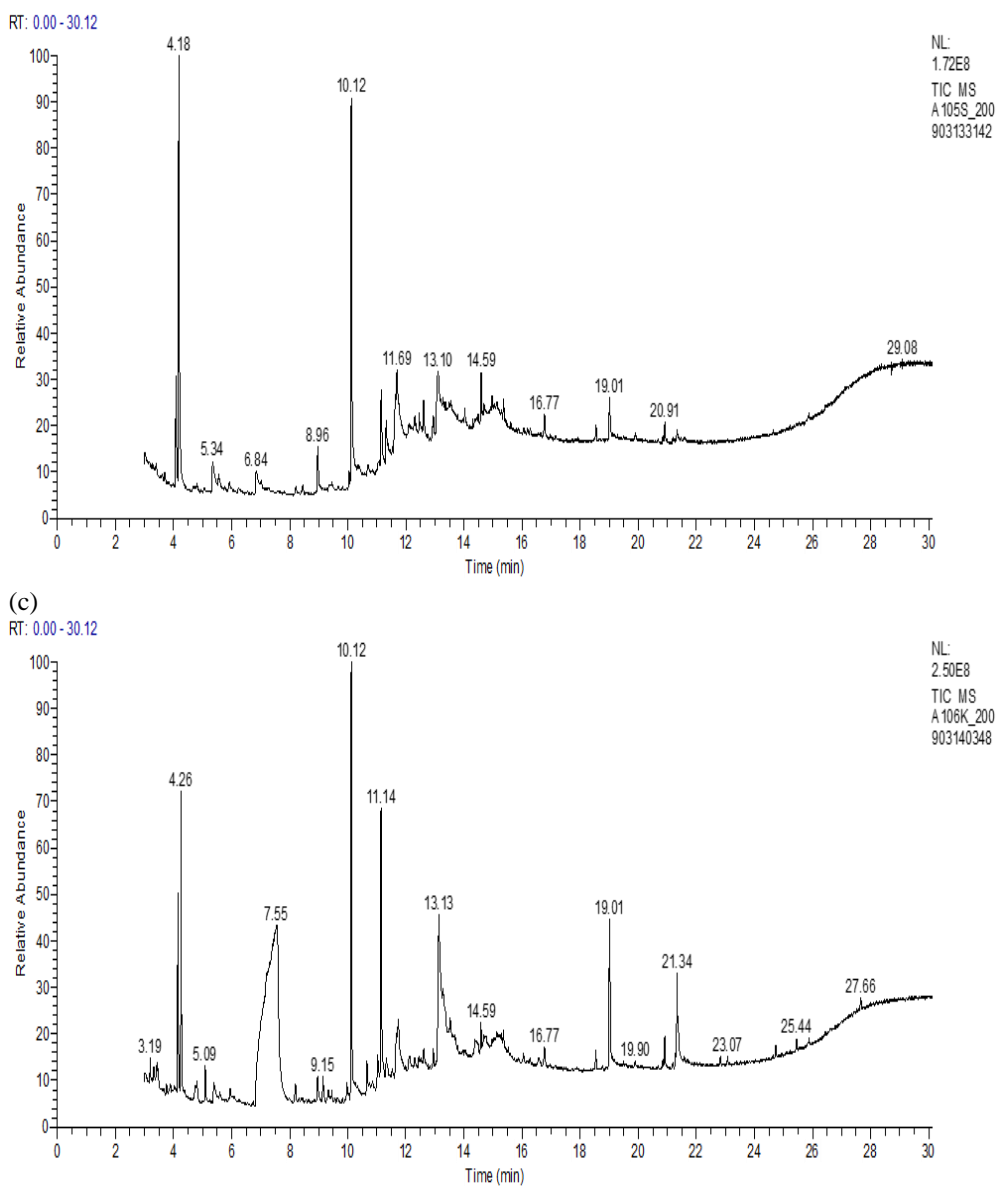
### 3.2 Identification of ECG Compound

Based on GC-MS's chemical analysis and library data, we found different peaks and retention times (R.T.) between the standard ECG and methanol extracts of fresh and dried RSFs (Figure 1a-c). From Figure 1a, the highest relative abundance of chemical compounds in the standard ECG is Dodecanoic acid methyl ester with 13.73 min R.T. In contrast, the most elevated close lot of chemical compounds fresh and dried methanol extracts RSFs have different R.T. (Figure 1b & c). In the methanol extract of fresh RSFs, 2,3-Butanediol, [S-(R\*, R\*)]- was detected at 4.18 min R.T. and 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- had 10.12 min R.T., which were more dominant than other chemical compounds. Moreover, the three highest relative abundance of chemical compounds was observed in methanol extract of dried RSFs were 2,3-Butanediol, [S-(R\*, R\*)]- with 4.26 RT, 4H - Pyran- 4 - one, 2, 3 - dihydro - 3, 5 - dihydroxy - 6 - methyl - with 10.12 RT and 5 -Hydroxymethylfurfural with 11.14 mins R.T.s. Unfortunately, we did not find any ECG compound among the three chromatograms.

Comparing to previous studies, the results of our RSFs extracts' GC-MS analysis differed.. According to a research study conducted in Malaysia, Tridecane, 4-methyl- compound has the highest peak with 13.75 min R.T. At the same time, GCMS analysis of RSFs extracts from Bogor Indonesian, Egypt, and India indicated that the highest peak was Nerylacetone at 4.43 min R.T., Methyl caproate at 7 min R.T. and 9-Octadecenoic acid (Z)-, methyl ester at 22 min R.T. respectively [20,21]. Despite limitations of GC-MS analysis in terms of the mass range and metabolites polarity, using the derivatization technique has expanded the range of separable and detectable chemical compounds in high-throughput profiling research. In addition, several artifacts caused by the derivatization reagent were observed in the GC-MS chromatograms. Therefore, N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) or N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) is often used for artefacts removal during derivatization [22].



(b)

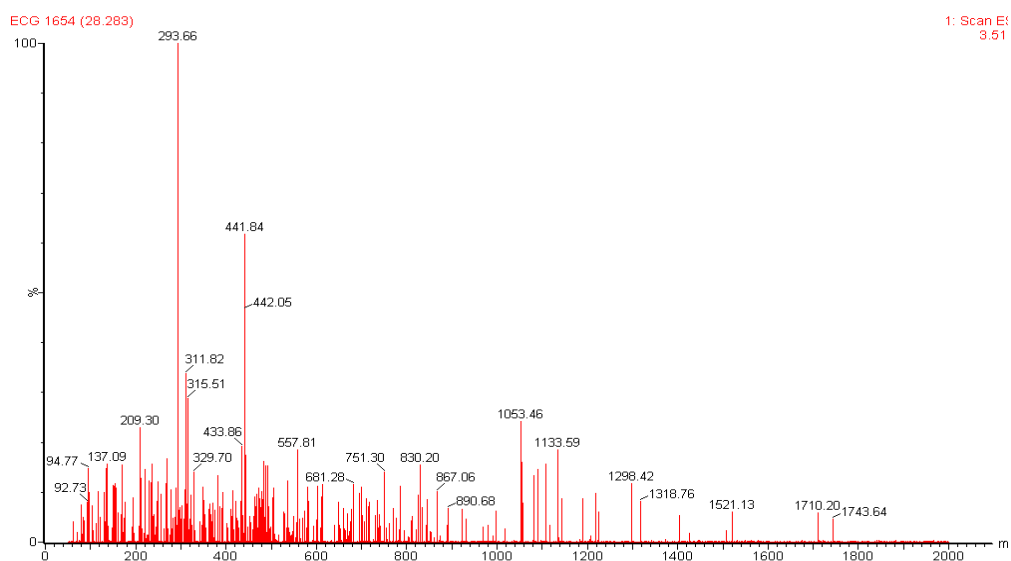


**Fig. 1.** GC-MS chromatogram results of methanol extract derived from ECG high purity (a), fresh (b), and dried RSFs (c).

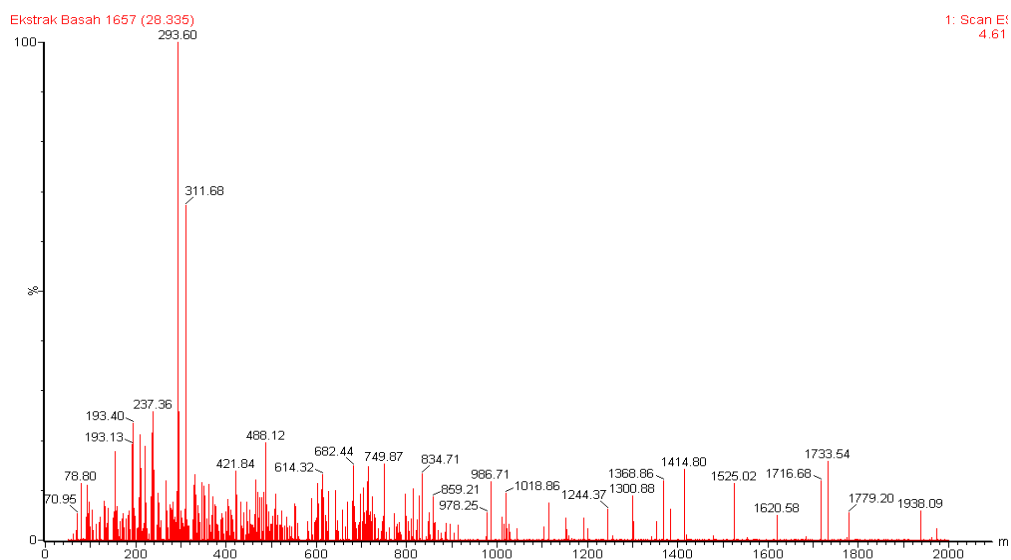
To identify ECG compounds in both RSFs extracts, we tried to analyze them using the LC-MS equipment (Figure 2a-c). The three highest peaks of ECG standard were observed at 293.66, 441.84, and 442.05 m/z, while fresh and dried RSFs extracts had the same peaks at 293.60 m/z. The other highest peak in fresh rSFs extracts were 311.68 and 237.36 m/z, whereas the dried

RSFs extracts had 293.73 and 294.69 m/z. based on the library of MassBank of North America (MoNA) [NP\_C2\_126\_p4\_G08\_POS\_iTree\_06], (-)-epicatechin 3-O-gallate has 447.0864 molecule weight and two fragmented ions [327.0553 and 295.0933 m/z] [23]. In previous reports, parent ion with 441 m/z corresponds to monomeric epicatechin gallate, identified as (-)-epicatechin gallate by direct comparison to  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data [24]. However, the parent ion with 441 m/z was low and was not detected in Figure 2b-c.

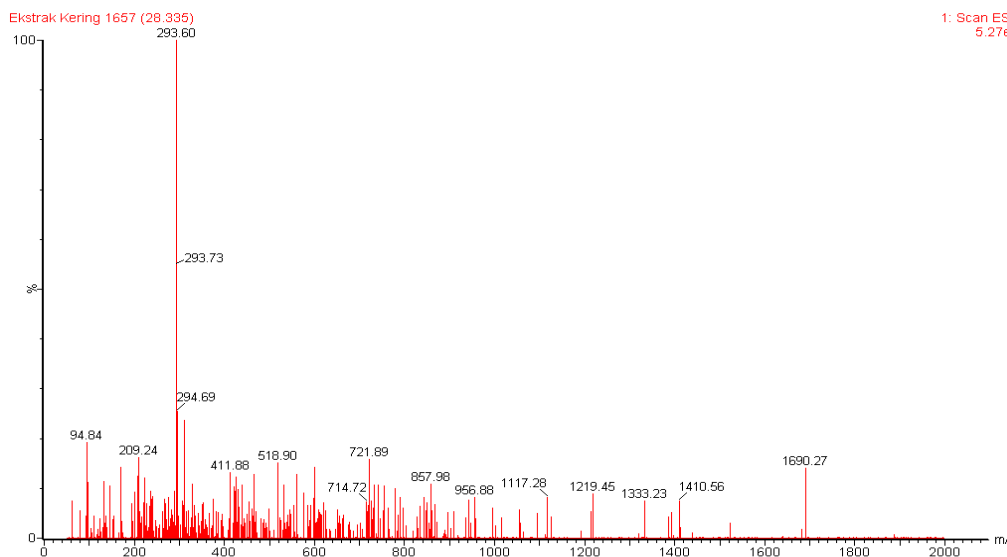
(a)



(b)



(c)



**Fig. 2.** Results of LC-MS analysis of (a) standard ECG, (b) fresh and (c) dried RSFs extracts analyzed using LC-MS.

Table 2 shows nine chemical compounds found in fresh and dried RSFs extracts. 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- and 2,3-Butanediol, [S-(R\*, R\*)]- were the highest proportion of chemical compounds (16.72 and 15.54 %) in the fresh RSFs extracts, followed by D-Glucose, 6-O-à-D-galactopyranosyl- (12.23 %) while the dried extracts were only glycerine (27.61 %). Other chemical compounds with both RSFs sections had a proportion of < 10 %.

The presence of compounds with pharmacological value was revealed by GC-MS analysis. As a result, fresh and dried RSFs extract yielded a total of 20 compounds. Based on previous studies, glycerine possess antibacterial activity [25]. 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl- has anti-oxidant, anti-microbial and anti-inflammatory activities [26]. 2,3-Butanediol, [S-(R\*, R\*)]- is a vicinal diol and chiral compound that can act as antifreeze agents. As a vital chemical, it can produce important derivatives, such as 1,3-butadiene and methyl ethyl ketone [27]. Honey contains a high level of isomaltose (D-Glucose,6-O-à-D-galactopyranosyl-), which when heated could improve immunosuppressive conditions. Heated isomaltose may have a therapeutic role in patients with compromised immunity as a result of chemotherapeutic agents [28].

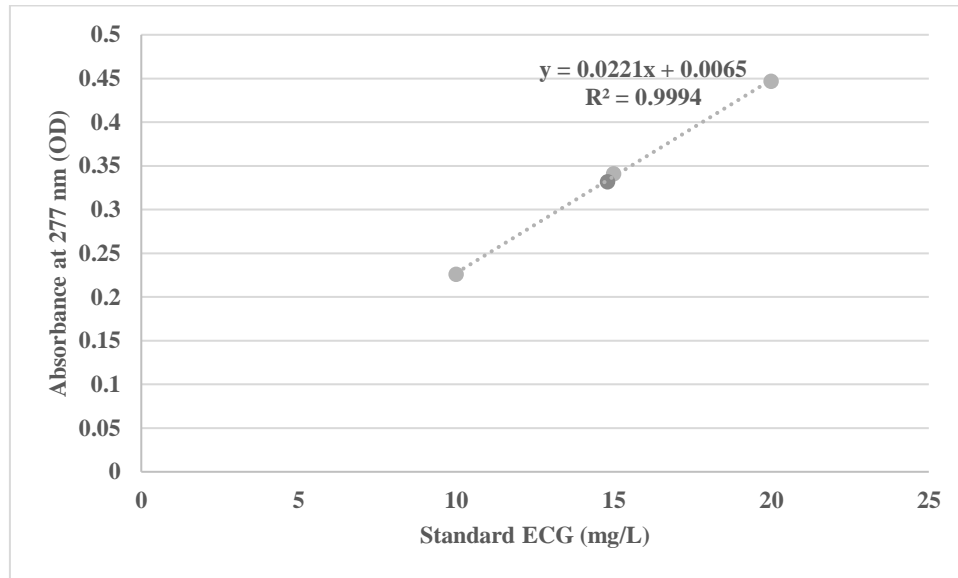
**Table 2.** Chemical compounds identified in fresh and dried RSFs extracts.

No	Retention Time (min)		Peak Height (mAU)		Area (%)		Nomenclature	Formula	M.W.	Activity
	Fresh	Dried	Fresh	Dried	Fresh	Dried				
1	4.18	4.26	160,932,511.21	164,595,000.46	15.54	4.59	2,3-Butanediol, (R*,R*)-	[S- C4H10O2	90	Antifreeze agents
2	6.84	7.55	8,876,450.23	85,412,847.24	2.41	27.61	Glycerin	C3H8O3	92	Antibacterial, as a moisturizer for rough, dry, itchy, scaly and irritated skin (e.g., skin burns from radiation therapy, diaper rash)
3	10.12	10.12	144,688,126.82	234,968,483.55	16.72	8.19	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	C6H8O4	144	Antimicrobial, anti-inflammatory, antioxidant activity
4	11.15	11.14	29,379,407.98	149,812,870.81	3.73	5.78	5-Hydroxymethylfurfural	C6H6O3	126	Antiproliferative inhibits the formation of sickled cells in the blood, active natural ingredient in nutraceuticals and dietary foods
5	11.69	12.13	29,549,604.02	8,576,113.84	12.23	0.77	D-Glucose,6-O-à-D-galactopyranosyl-	C12H22O11	342	Melibiose, the reduction of the intracellular concentration of toxic sugars or sugar metabolites
6	13.09	13.28	21,790,168.80	37,786,571.98	7.05	4.64	Sucrose	C12H22O11	342	Source of energy



7	14.96		11,219,522.35		3.78		Cyclooctasiloxane, hexadecamethyl-	C16H48O8Si8	592	Antimicrobial
8	19.01	19.01	16,318,537.00	79,936,279.59	2.24	3.74	n-Hexadecanoic acid	C16H32O2	256	Palmitic acid, antiviral, anti-inflammatory, antibacterial, antimicrobial, hypocholesterolemic, nematocide, pesticide, hemolytic, lubricant, 5- $\alpha$ -reductase inhibitor
9		13.13		78,414,479.65	-	7.32	4H-Pyran-4-one,5-hydroxy-2-(hydroxymethyl)-	C6H6O4	142	Kojic acid, catechol oxidase inhibitor, antibiotic, antioxidant, skin lightening agent

### 3.3 Quantification of ECG Compound



**Fig. 3.** A standard curve of standard ECG measured using a spectrophotometer at 277 nm.

**Table 3.** Quantification of ECG compound in fresh and dried RSFs extracts.

	Extract concentration (mg L <sup>-1</sup> )	ECG concentration (mg L <sup>-1</sup> )	<i>p</i>
Fresh	100	1.65 ± 0.25	
Dried	100	6.2 ± 0.6	0.002

The mean data are presented ± S.D. Significant differences between fresh and dried samples were determined using an independent t-test. *P*-value indicates a substantial difference.

Further analysis using a spectrophotometer was used to quantify the ECG concentration in fresh and dried RSFs extracts [Figure 3 and Table 3]. A linear regression formula  $y=0.0221x + 0.0065$ , generated using some diluted standard ECGs, was needed to calculate ECG concentration in both extracts. Fresh and dried RSFs extracts had  $1.65 \pm 0.25$  % and  $6.2 \pm 0.6$  % ECG concentrations. Statistically, the ECG concentration in dried RSFs extracts was significantly higher than the ECG concentration in fresh RSFs extracts with  $P=0.002$ . Based on the authors' knowledge, we firstly reported the ECG concentration in fresh and dried RSFs extracts. By water removal in the dried RSFs extract, the ECG concentration increases 3.76 times higher than fresh RSFs extract. Another reason for increased ECG concentration in dried RSFs extracts is that the ECG compound has thermal stability during the drying process [29].

## 4 Conclusion

Extraction of fresh RSFs has a greater yield than the extraction of dried RSFs. However, ECG compound is detected in dried RSFs extract, consisting of different phytochemicals from fresh RSFs extract. In addition, the dried RSFs section has a  $6.2 \pm 0.6$  mg/L ECG compound, which is more significant than the fresh RSFs extract ( $1.65 \pm 0.25$  mg/L). Therefore, it becomes a potential herbal drug for diabetes patients against the high expression of SGLT2 protein.

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