The Influence of pH and Temperature on The Stability of Catechin Isolated from Gambier (*Uncaria gambir* (Hunter) Roxb

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ABSTRACT

The pre formulation data is necessary to develop a pharmaceutical dosage form. Information on the degradation processes and/or the stability of drug substance is one of important data needed. So far, data on the stability of catechin are still limited. Aims of the research were to isolate of catechin from gambier (*Uncaria gambir* (Hunter) Roxb and to evaluate the effect of pH and temperature on the chemical stability of catechin. Catechin was isolated by maceration method using ethyl acetate and continuing with hot distilled water. The chemical stability study was performed in buffer solutions at the concentration of 0.1 M and ionic strength of 0.3 at variables pH of 1.5, 7.4, and 8.0, and at temperatures of 25, 37, and 55 °C. The stability of catechin was observed after storage time of 10 days. The percentage of catechin remained was determined by reverse phase High Performance Liquid Chromatography (HPLC) using C-18 column and mobile phase consisted of acetonitrile-distilled water-formic acid (18:81:1 v/v/v). The flow rate was adjusted at 1 mL/min. Catechin was detected by UV-Vis detector at maximum absorption wavelength of 279 nm. Results showed that the mean percentage of catechin remained were decreased within buffer solution at pH of 1.5, 7.4, and 8.0 at various temperatures. The percentage of catechin remained within buffer solution of pH 1.5 at temperatures of 25, 37, and 55 °C were 82.80, 76.08, and 58.96%, respectively. The percentage of catechin remained within buffer solution of pH 7.4 at temperature of 25, 37, and 55 °C were 50.26, 26.16, and 17.01%, respectively. The higher of the pH of buffer solution, the higher the decomposition of catechin ($p < 0.01$). Catechin was more stable in acidic compared to base solution.

Keywords: stability, catechin, gambier.

INTRODUCTION

Uncaria gambir Roxb. or gambier is one of plant in the Rubiaceae family. It contains an officially recognized pharmacological compound (Heitzman *et.al*, 2005). It is an agricultural commodity that has great potential for trading and has been widely used for a long time, both in traditional use and developing with more modern management.

Gambir is a type of compacted sap obtained from processing the leaves and twigs of the gambier plant (Nazir, 2000). Gambier plants (*Uncaria gambir*) are widely available in Southeast Asia, including Indonesia, in the form of shrubs that have twisted hard stems, short-stemmed leaves and light green color. This plant has been widely used by the community in medicine such as diarrhea, gingivitis and throat inflammation, adstringents, burns medicine, mouthwash, mouth ulcers, and fragrances (Bakhtiar, 1991).

This plant contains various components, one of which is catechins. Catechins are a type of tannin in the form of polyphenols which have supplemental properties and are widely found in plants. Young leaves contain 10-18% more catechins than old leaves. The physiological functions of catechins include: antioxidants, antibacterials, cholesterol control and absorption of UV light (Miller, 1996).

Catechins can form dimers each other called pro anthocyanins. This type of tannin is easily oxidized as indicated by a changing in color. Prolonged storage can cause deposits, known as phlobaphenes or phlobatnins (Mills & Bone, 2000). Catechins are included in the phenolic group that has low stability in alkaline solutions and experience faster decomposition in alkaline pH solutions (Gotti *et.al*, 2004).

The content of catechins from Gambir provides good health benefits so that its use as a medicinal ingredient is growing. However, before catechins are formulated into medicinal dosage forms, their pre formulation needs to be reviewed so that they can be used to create suitable formulations. One of the pre formulation data that can be used in characterizing a drug compound is stability data, both in solution and in a solid state. This stability can be affected by temperature, hydrolysis, oxidation, photolysis, pH etc. (Connors, 1992; Wells, 1998). Therefore, it is necessary to discover the stability profile of catechins in solution form against the influence of pH and temperature during certain storage.

METHODS

Equipments and Materials

Shimadzu High Performance Liquid Chromatography (HPLC) consisting of: LC-10AT VP pump, UV-Vis detector (SPD-10A VP Shimadzu), control system (SCL-10A VP Shimadzu), 100 µL syringe (SGE Australia), column C-18 (CLC-ODS M), Millipore filter paper, digital scale, pH meter (Griffin), Ballenkamp incubator, HAAKE F3 Sisons water bath, ultrasonic, oven, refrigerator, Amberlite XAD4 resin column, vacuum filter, amberglass vial and glassware commonly used in the laboratories.

Materials used: Gambir, standard catechins, Amberlite XAD4 resin, Sephadex LH-20, potassium dihydrogen phosphate, potassium hydrogen phosphate, phosphoric acid, distilled water were ordered from commercial source, and organic solvent usual used.

Isolation of Catechin from Gambir

One kilogram of gambier is macerated with ethyl acetate solvent and then it is filtered in vacuo to obtain a thick ethyl acetate extract. Hot water was added to the extract and passed into the Amberlite XAD4 resin column. The filtrate obtained was dried then added with methanol and passed to the Sepahdex LH-20 column, then dried to obtain catechin powder. The catechins were compared with standard catechins using High Performance Liquid Chromatography (HPLC), the C-18 column stationary phase and the mobile phase consisted of acetonityl-water- formic acid (18: 81: 1 v / v / v) with a regulated flow rate of 1 mL/minute.

Evaluation of Catechin Raw Material

Catechin examination includes solubility in water, 6% ethanol, and ethyl acetate; organoleptic examination, and determination of the melting distance (Windholz, 1983).

Determination of Catechin Solubility

The solubility of catechins was determined using spectrophotometric method. The maximum absorption wavelength of the catechin standard curve in water, ethanol, and ethyl acetate solvents was determined. 50 mg carefully weighed were dissolved in each solvent (water, 96% ethanol, and ethyl acetate) up to 50 mL to obtain a main solution concentration

of 1 mg/mL. The standard solutions in each solvent were prepared at concentrations of 20, 30, 40, 50, and 60 µ/mL. The absorption of solution was measured using a UV spectrophotometer at the maximum absorption wavelength of each solvent. The calibration curve is created based on the absorbance of each standard solution.

One gram of catechins was poured into 10 mL of distilled water. The mixture was stirred using a magnetic stirrer for 3 hours until equilibrium reached, then filtered. The filtrate obtained was diluted with water to obtain a solution absorption ranging from 0.2 to 0.8. Catechin levels were calculated using a standard curve. The determination of the solubility of catechins in ethanol 96% and ethyl acetate was carried out the same as for the determination of the solubility of catechins in distilled water.

Catechin Stability Study

Determination of catechin levels at various pH and temperatures after 10 days of storage time

181.25 mg of catechins were weighed and a catechin solution with a concentration of 2.5x 10-3 M was prepared in a buffer solution of pH 1.5 using a 250 mL volumetric flask. 20 mL of the solution is pipetted and placed into the Amberglass vial. For t = 0 days, the catechin solution in the vial was injected directly into the HPLC system. Catechin solutions in other vials were stored at 25, 37, and 55 °C for 10 days then injected into the HPLC system. The same procedure was performed at pH 7.4 and 8. The flow rate was adjusted to 1 mL/minute.

Catechin solutions were treated at various temperatures and pH within 10 days of storage. Catechin levels in the stability test were measured using High Performance Chromatography (HPLC) using a stationary phase column C-18 with a column length of 25 cm and a mobile phase consisting of acetonitrile-distilled water-formic acid (18:81:1 v/v/v) at a flow rate of 1 mL/min and the pH of 2.5 (31). A correlation curve between catechin concentration and peak height was established.

Preparation of Calibration Curve

145.14 mg of catechins was weighed to make catechin main standard solution at a concentration of 10^{-2} M in the mobile phase in a 50-mL volumetric flask. Then dilution was carried out to obtain 7 standard solutions at levels of 0.5, 1, 1.5, 2, 2.5, 3, and 3.5 x 10^{-3} M. Each standard solution was injected into the reverse phase HPLC system using mobile phase acetonitrile-distilled water-formic acid (18:81:1 v/v/v). The absorption of solution was detected at a maximum absorption wavelength of 279 nm. The flow rate was adjusted at a rate of 1 mL/minute. Calibration curve was created.

Determination of Catechin Levels at Various Temperature and pH

181.25 mg of catechins weighed carefully was dissolved in a buffer solution of pH 1.5 in a 250 mL volumetric flask. 20 mL of the solution was pipetted and put into the amberglass vial. For t = 0 days, the catechin solution in the vial was injected directly into the HPLC system. Catechin solutions in other vials were stored at 25, 37, and 55°C for 10 days then injected into the HPLC system. The same procedure was performed at pH of 4 and 8. The flow rate was adjusted to a rate of 1 mL per minute.

Statistical Analysis

The influence of pH and temperature on the catechins remaining in solutions were analyzed statistically using Two-way ANOVA and Pearson Correlation.

RESULTS AND DISCUSSION

Results of the examination of catechin raw materials including solubility, melting range point, organoleptic, and maximum absorption wavelength can be seen in the following Table 1. The solubility of catechins in water obtained was rather difficult to dissolve, in 96% ethanol it was soluble, and in ethyl acetate it was readily soluble.

Table 1. Results of Catechin Raw Material Evaluation

No.	Examination	Requirement (Windholz, 1983)	Observation
1.	Solubility		
	- In distilled water	Sparingly soluble	Sparingly soluble (1:92)
	- In ethanol	Soluble	Soluble (1:18)
	- In ethyl acetate	Freely soluble	Freely soluble (1:4.2)
2.	Melting range	175-177 °C	169-172 °C
3.	Organoleptic		
	- Form	-	Powder
	- Color	-	Brownish white
	- Odor	-	Specific
4.	Ultraviolet spectrum in ethyl acetate solution	λ_{max} = 279 nm	λ_{max} = 279 nm

The regression equation of the catechin solution calibration curve in the mobile phase of acetonitrile-distilled water-formic acid (18:81:1 % v/v/v) obtained was Y = (0.27x105) + (1800x105) X and the value of coefficient correlation, r, was 0.994. The precision, standard deviation and coefficient of variation obtained were 1.0065 x 10^{-3} and 0.6%, respectively (n = 6).

Catechins dissolved in buffer solutions at various pH and stored for 10 days at various temperatures were decomposed. It can be observed from the changing in the color of the catechin solution. On day 0, the solution was clear and after being stored for 10 days, the catechin solution becomes brownish and after stored for longer time, the color of the solution will turn dark brown. This is due to the non-enzymatic oxidation of catechins, resulting in color changes in just a few hours (Lopez-Toledano *et.al*, 2002). Catechins are weakly acidic and tend to be stable in acidic solutions (pH <5) (Zhu *et.al*, 2002).

Catechins are flavan-3-ol compounds derived from flavonoids that are easier to decompose in alkaline solutions. The decomposition will be directly proportional to the storage time because the longer the catechins are stored, the more they will decompose. From previous research reports it was known that catechins had an average pKa value of 6.13. Therefore, catechins are more ionized in alkaline solutions so they are easier to decompose (Baweja, 1986).

The instability of catechins in alkaline solutions is catalyzed by hydroxyl ions which dominate at high pH causing the oxidation rate in the solution to be faster. From the catechin structure, it is known that catechins contain many hydroxyl groups and the catechol structure of the B ring is a group that is sensitive to oxidation. This oxidation process causes the catechin structure to be oxidized into quinones so that the solution was brown (Danilewicz, 2003).

The breakdown of catechins also occurs due to epimerization of catechins to form epicatechins which are relatively unstable in alkaline pH solutions but qualitatively, analysis using the HPLC system shows that catechins and epicatechins have the same retention time so that the change of catechins to epicatechins or vice versa is difficult to distinguish (Zhu *et.al*, 2002: Zhu & Hammerstone, 2003).

Temperature can also increase the rate of the reaction to degrade catechins in the solution. The higher the temperature used, the more catechins will be broken down. In this study, the levels of catechins dissolved in buffer solutions at pH of 1.5, 7.4, and 8 will decompose more rapidly with an increasing of temperature. Increasing the temperature will increase the rate of decomposition of a drug compound. At high temperatures, the number of collisions between reacting molecules per unit time will increase so that the reaction rate increases. The effect of temperature on the rate of decomposition is illustrated by the Arrhenius equation. Temperature also affects the buffer solution. Most alkaline buffers change more easily than acidic buffers due to the influence of temperature (Martin *et.al*, 1983; Wells 1998).

The chromatogram showed the decomposition of catechins, this was indicated by the presence of several new peaks formed after catechin solutions at pH 1.5, 7.4, and 8 stored for 10 days. So far, no research has been found that explains the types of compounds that are formed from the breakdown of catechins. However, when viewed from the catechin structure, it is possible that the compounds formed come from breaking the catechin chain.

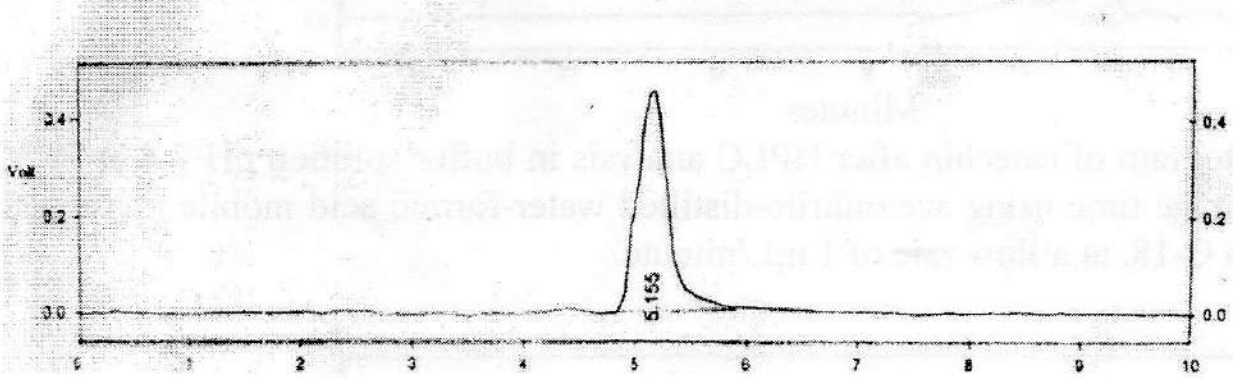

Figure 1a. Standard catechin chromatogram using acetonitrile-water-formic acid mobile phase (18: 81:1 v/v /v), column C-18, at a flow rate of 1 mL/min.

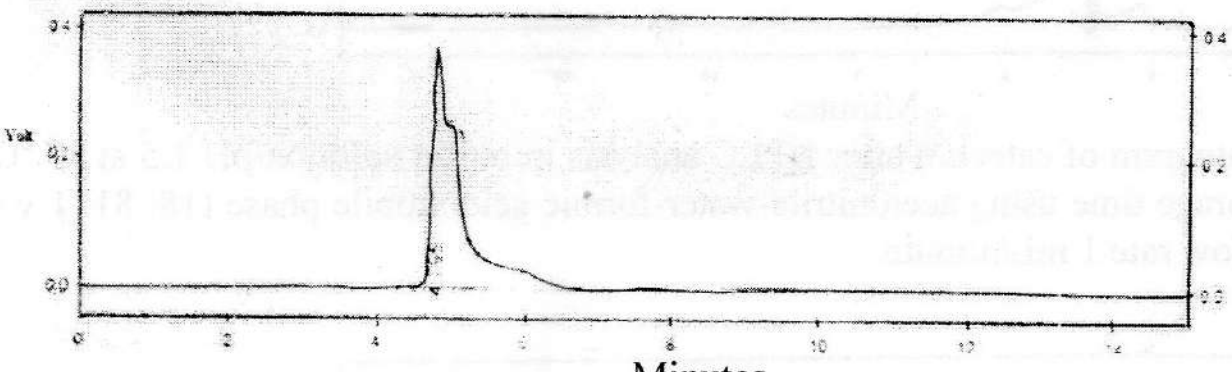

Figure 1b. Chromatogram of catechin after HPLC analysis in buffer solution pH 1.5 at 25 °C after 10 days of storage time using acetonitrile-distilled water-formic acid mobile phase (18: 81: 1 v/v/v), column C-18, at a flow rate of 1 mL/minute.

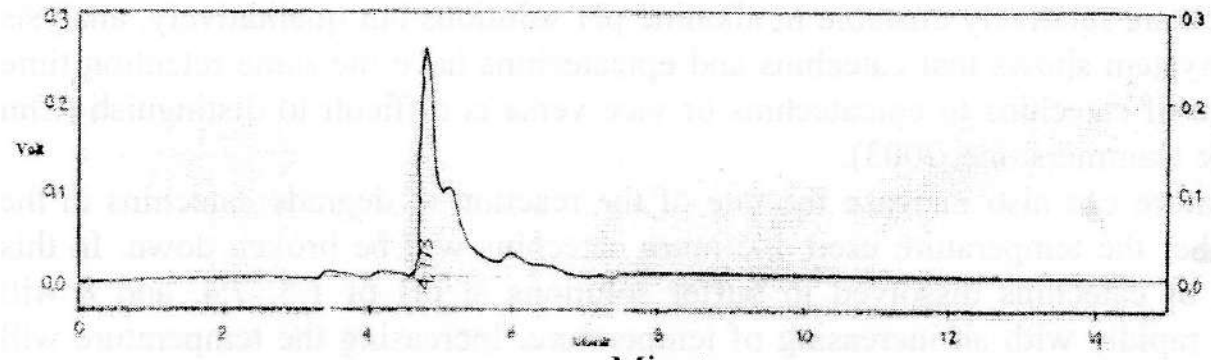

Figure 1c. Chromatogram of catechin after HPLC analysis in buffer solution pH 7.4 at 25 °C after 10 days of storage time using acetonitrile-distilled water-formic acid mobile phase (18: 81: 1 v/v/v), column C-18, at a flow rate of 1 mL minute.

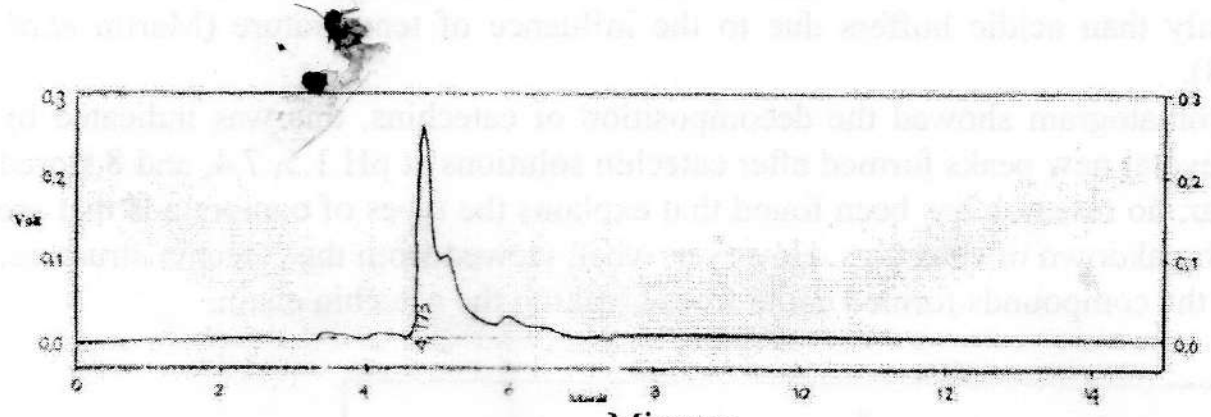

Figure 1d. Chromatogram of catechin after HPLC analysis in buffer solution pH 7.4 at 37 °C after 10 days of storage time using acetonitrile-distilled water-formic acid mobile phase (18: 81:1 v/v/v), column C-18, at a flow rate of 1 mL/minute.

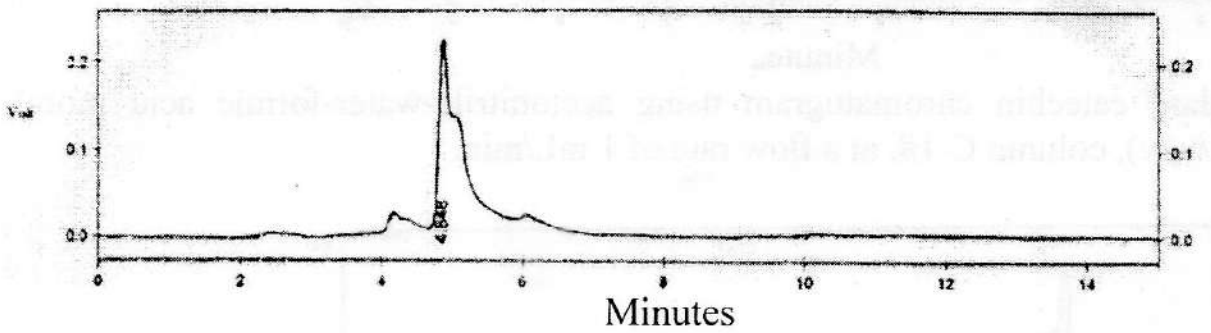

Figure 1.e. Chromatogram of catechin after HPLC analysis in buffer solution pH 1.5 at 37 °C after 10 days of storage time using acetonitrile-water-formic acid mobile phase (18: 81: 1 v / v), column C-18, flow rate 1 mL/minute.

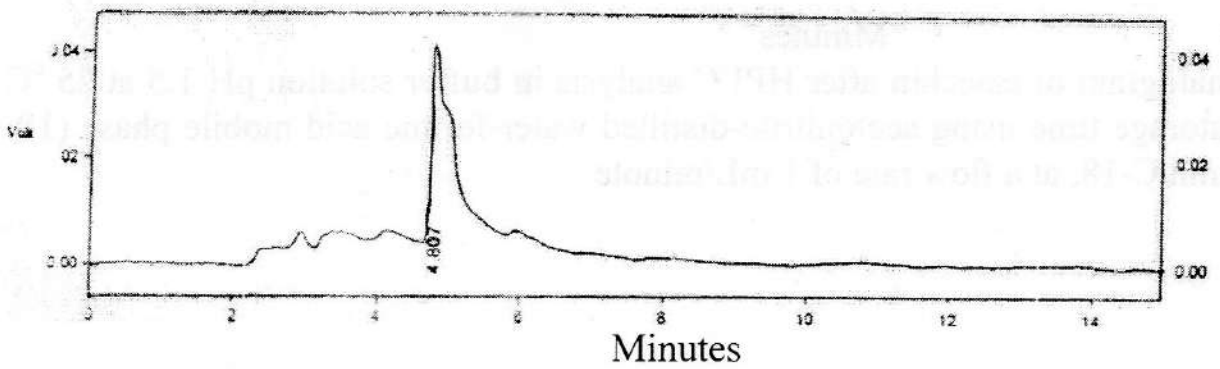

Figure 1.f. Chromatogram of catechin after HPLC analysis in buffer solution pH 1.5 at 55 °C after 10 days of storage using acetonitrile-water-formic acid mobile phase (18: 81:1 v/v/v), column C-18, flow rate at 1 mL/minute.

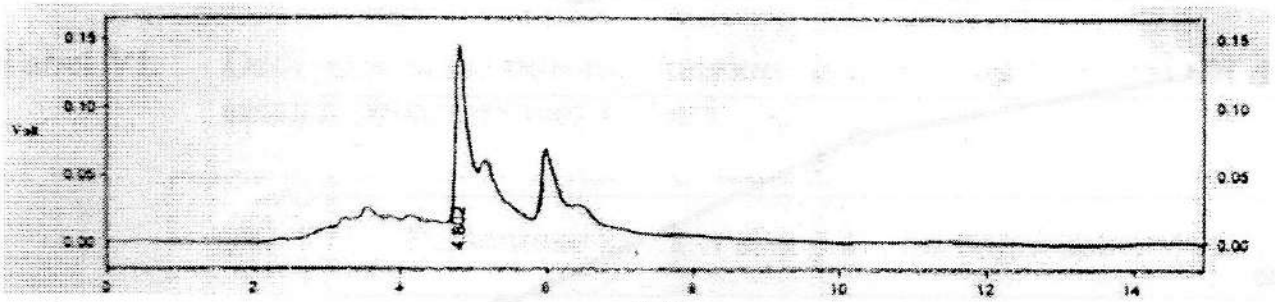

Figure 1g. Chromatogram of catechin after HPLC analysis in buffer solution pH 7.4 at 55 °C after 10 days of storage using acetonitrile-water-formic acid mobile phase (18: 81:1 v/v/v), column C-18, at a flow rate 1 mL/minute.

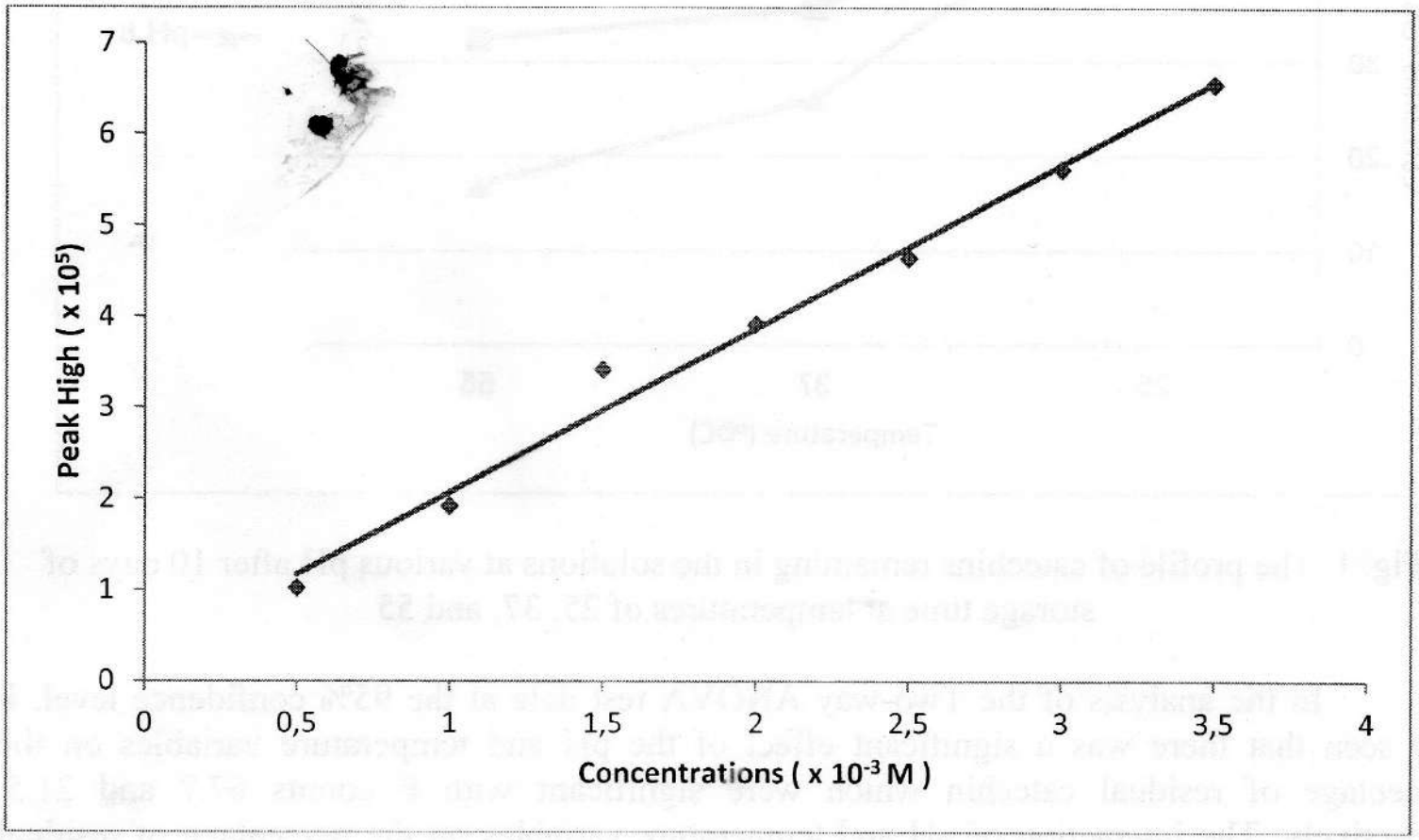

Figure 2. Curve of standard catechins in mobile phase acetonitrile-distilled water-formic acid (18: 81:1 v/v/v), column C-18, at a of flow rate 1 mL/min.

Percentage of catechins remaining in buffer solutions at pH of 1.5 after stored for 10 days at 25, 37, and 55 °C, were 82.80 ± 0.15, 76.08 ± 2.39, and 58.96 ± 1.82 %, respectively. The percentage of catechins remaining in buffer solutions pH 7.4 stored for 10 days at 25, 37, and 55 °C were 50.26 ± 0.52, 35.78 ± 2.34, and 32.48 ± 0.15%, respectively. The percentage of catechins remaining in buffer solutions of pH 8.0 which were stored for 10 days at 25, 37, and 55 °C were 50.69 ± 0.46, 26.16 ± 2.11, and 17.01 ± 0.47 %, respectively.

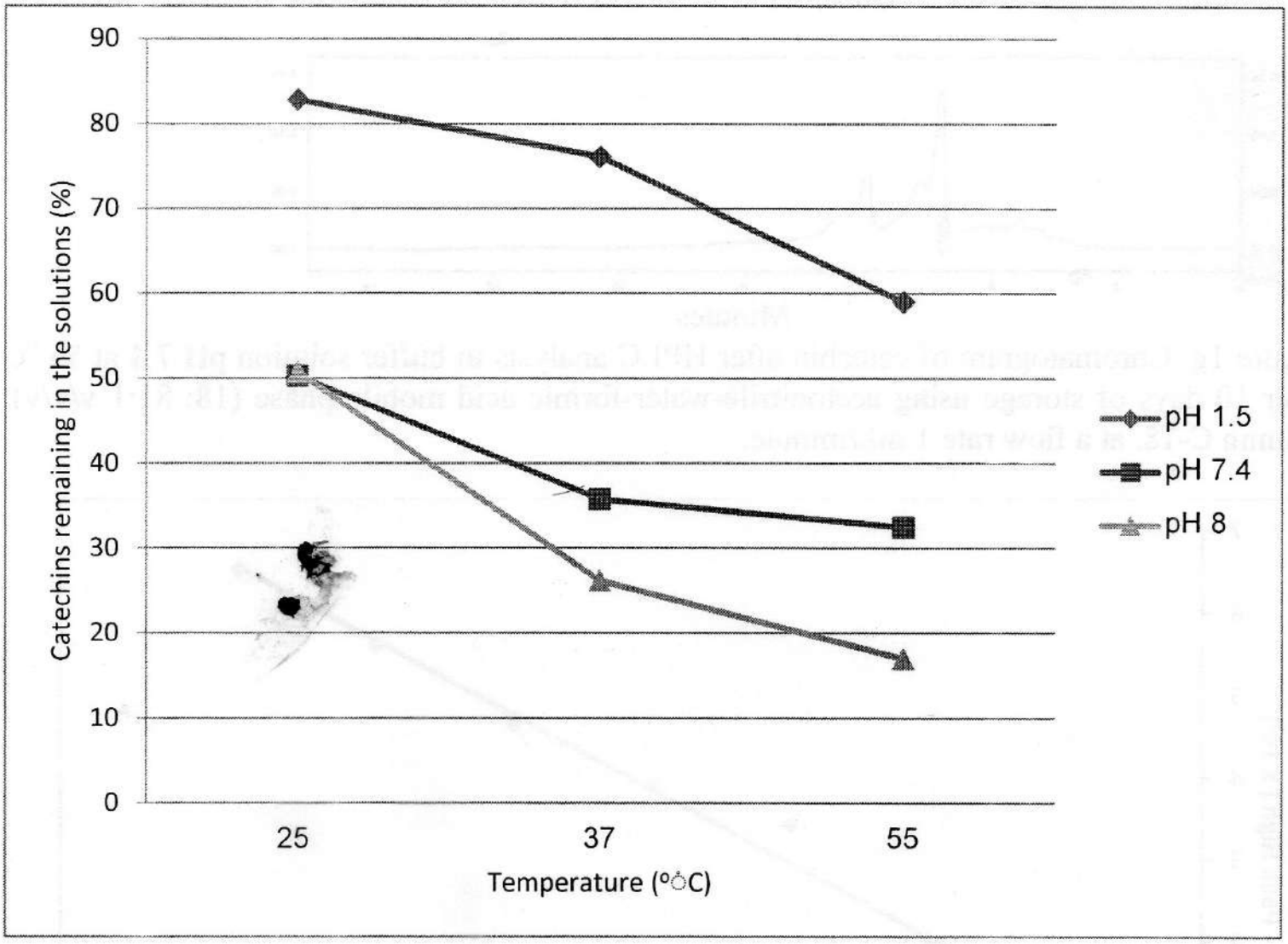

Fig. 1. The profile of catechins remaining in the solutions at various pH after 10 days of storage time at temperatures of 25, 37, and 55

In the analysis of the Two-way ANOVA test data at the 95% confidence level, it was seen that there was a significant effect of the pH and temperature variables on the percentage of residual catechin which were significant with F counts 67.7 and 21.5, respectively. The interaction of pH and temperature variables on the percentage of residual catechins gave an F value of 2.1.

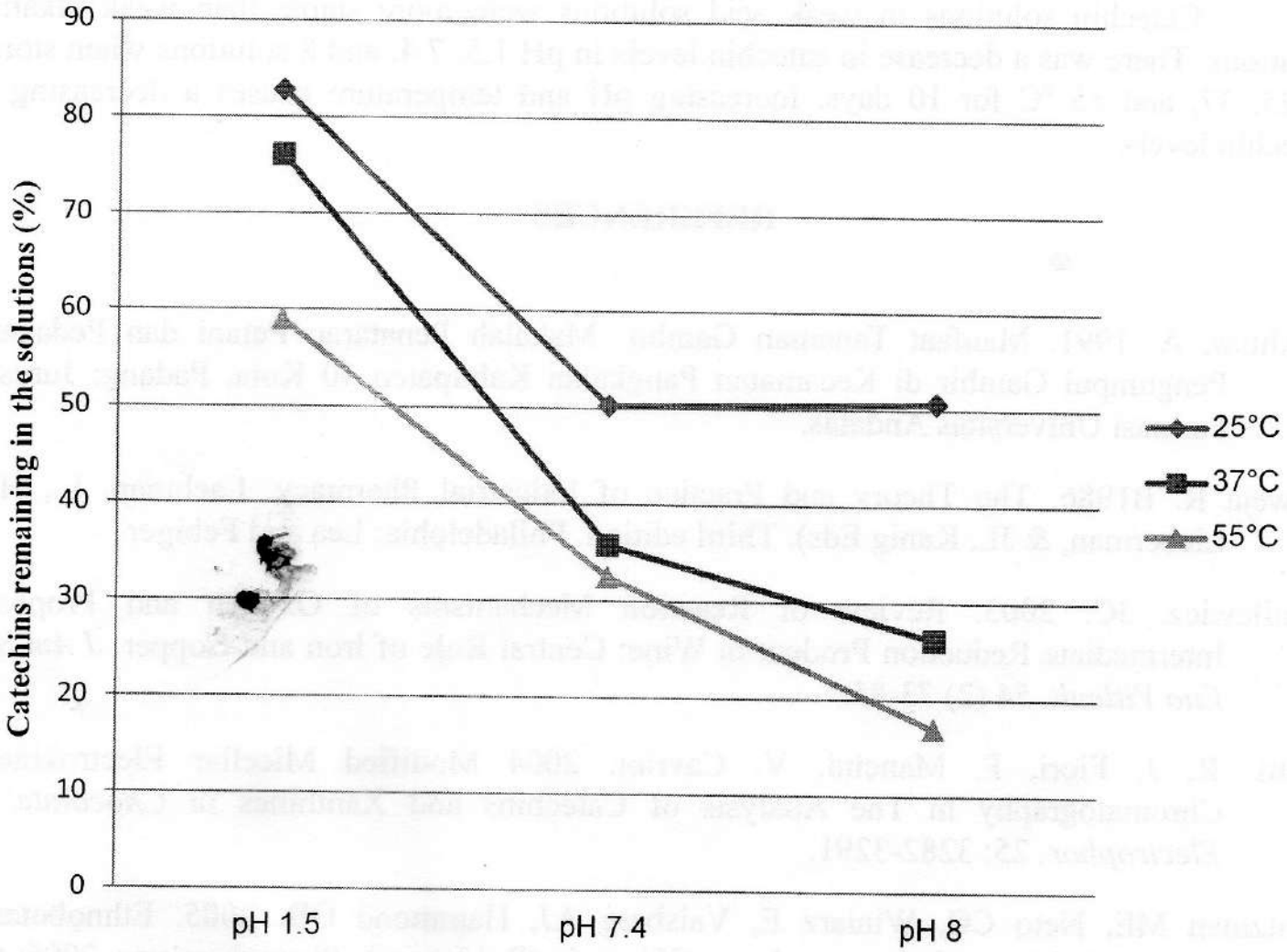

Fig. 1. The profile of catechins remaining in the solutions at various temperatures after 10 days of storage time at pH of 1.5, 7.4, and 8.

After the bivariate correlation analysis using the Pearson Correlation test, it was known that the correlation coefficient value is -0.767 between the pH variable and the variable percentage of residual catechins. While the Pearson correlation coefficient, the relationship between temperature and the percentage of catechins left is -0.415. The probability value less than 0.05 indicates a significant effect of pH and temperature variables on the percentage of residual catechins. The negative sign indicates the opposite relationship. The results of this analysis indicate an inversely proportional relationship between pH and temperature on the stability of catechins. The higher the pH and temperature of the catechin solution, the less catechin stability and residual catechin levels will be.

CONCLUSION

The percentage of catechins remaining in buffer solutions at pH of 1.5 which was stored for 10 days at 25, 37, and 55 ^{o}C were 82.80 ± 0.15, 76.08 ± 2.39, and 58.96 ± 1.82%, respectively. Meanwhile, the percentage of catechins remaining in the buffer solution pH 7.4 stored for 10 days and the same temperature were 50.26 ± 0.52, 35.78 ± 2.34, and 32.48 ± 0.15%, respectively. Whereas at pH 8.0 which was stored at the same time and temperature intervals, the percentage of catechins remaining in the buffer solution were 50.69 ± 0.46, 26.16 ± 2.11, and 17.01 ± 0.47 %, respectively.

Catechin solutions in weak acid solutions were more stable than weak alkaline solutions. There was a decrease in catechin levels in pH 1.5, 7.4, and 8 solutions when stored at 25, 37, and 55 °C for 10 days. Increasing pH and temperature causes a decreasing in catechin levels.

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