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Formulation and validation of analytical methods for ursolic acid in *Plantago major* gel preparations

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ABSTRACT: *Plantago major* of the plantain family has been observed as a wound-healing plant species on account of its ursolic acid (UA) content. The study's objectives were twofold: to prepare UA gels from *P. major* and validate the analytical method using TLC-densitometer. Here, vacuum and open column chromatography were used to isolate UA from the methanol extract of *P. major*. Afterward, data on the compound's physical properties and infrared (IR) spectroscopy, electrospray ionization mass spectrometry (ESI-MS), and Nuclear Magnetic Resonance (^{1}H NMR, ^{13}C NMR) results were analyzed. Carbomer and hydroxy propyl methyl cellulose (HPMC) were used as gelling agents to make the UA gel. To validate the analytical method, TLC-densitometer was performed using TLC silica gel 60 F_{254} plates (the stationary phase) and a mixture of toluene: ethyl acetate: formic acid (8:2:0.1, the developing solvent). The results showed that the UA gel was successfully prepared using both gelling agents and that its physical properties (i.e., visual forms, viscosity, and flow behavior) and pH values were maintained throughout the 90-day accelerated stability test (40±2°C; 75±5% RH). In addition, the method validation results indicated compliance with relevant parameters: linearity (r=0.998), limit of detection (4.55 ng/spot) and limit of quantification (15.17 ng/spot), recovery (95.04-116.82%), intraday precision (RSD=1.24–1.96%) and interday precision (RSD=6.29%). Overall, the UA gel is stable for 90 days (accelerated stability test), and the TLC-densitometry proposed in the research can be used to identify and measure the chemical content of a gel preparation.

KEYWORDS: Gel; method validation; *Plantago major*; plantain; stability test; ursolic acid

1. INTRODUCTION

Ursolic acid (UA, Figure 1), a triterpenic acid, can be found in significant amounts in the leaves and berries of many medicinal herbs like *Arctostaphylos uva-ursi* (bearberries), *Calluna vulgaris, Eriobotrya japonica, Eugenia jambolana, Ocimum sanctum, Rhododendron hymenanthes Makino, Rosemarinus officinalis, Vaccinium macrocarpon* (cranberries) and the natural wax layers of apples, pears, prunes, and other fruits [1]. Its occurrence in *Plantago major* has also been scientifically documented [2-5]. In addition, ursolic acid has been reported to show various pharmacological activities, including immunomodulation, anti-inflammatory, and cholinesterase inhibition [6-8], and prevention and treatment of different types of cancers [2, 4, 6, 7, 9-14]. Furthermore, UA has cytotoxic activities on A549, HeLaS3, Hep G2, KB, MCF-7, MDA-MB-231, and SiHa cancer cell lines and inhibits IL-1β, IL-6, IFN-γ, and TNF-α production [3, 15]. UA-loaded lipid-core nanocapsules and UA isolated from *Shorea robusta* exhibited wound healing activity in mice [16, 17].

UA can be transformed into topical wound-healing preparations like gels and creams. Direct application to wounds assures that the active compounds are delivered fast to the sites of action. Gels are considered a delivery system suitable for drugs because they have the optimal cutaneous drug delivery, contain very little grease, and are easily removable from the skin. Not only safety and efficacy aspects should be accomplished by drug products, but also a quality aspect. Therefore, physical and chemical stability tests are necessary. Prior to the chemical stability test, it is essential to develop and validate the analytical method. Various analytical techniques such as GC-MS and HPLC were conducted to measure the UA content [18, 19]. However, because these methods are costly and require specialized expertise, the TLC-densitometric method was used in the UA analysis [3, 20, 21]. Moreover, this method was able to separate UA from its isomer commonly found in herbs, oleanolic acid, after simple pre-derivatization with iodine [22]. The study was

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designed to formulate UA into gels, evaluate the gel's pH and physical stability, and validate the TLC-densitometric method proposed to determine the UA concentrations in the gels.

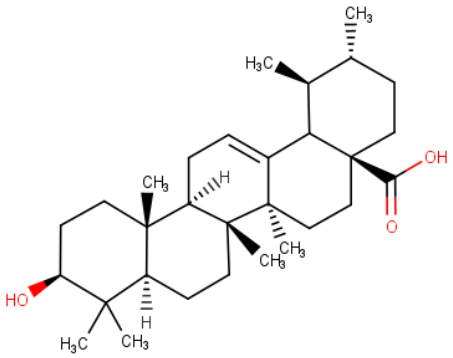

Figure 1. Chemical structure of ursolic acid

2. RESULTS AND DISCUSSION

2.1. Ursolic acid isolation and identification

The isolated compound had white amorphous powder, a melting point at 252.4-254.4°C, UV λ_{max} (MeOH) 207.5 nm. IR ν_{max} (KBr) 3418.42, 2927.19, 1694.55 cm^{-1}; ESI-MS m/z 479.3518 $[23]^+$; ^{1}H-NMR (DMSO-d_5) δ 2.98 (1H, br t, H-3), 5.10 (1H, br s, H-12), 2.06 (1H, d, J = 11.2, H-18), 0.87 (3H, s, H-23), 0.65 (3H, s, H-24), 0.84 (3H, s, H-25), 0.72 (3H, s, H-26), 1.01 (3H, s, H-27), 0.78 (3H, d, J = 6.3, H-29), 0.89 (3H, s, H-30) ; ^{13}C-NMR (DMSO-d_5) δ 38.3 (C-1), 27.0 (C-2), 77.0 (C-3), 39.5 (C-4), 54.9 (C-5), 18.1 (C-6), 30.3 (C-7), 40.3 (C-6), 47.1 (C-9), 36.6 (C-10), 23.9 (C-11), 124.7 (C-12), 138.3 (C-13), 41.7 (C-14), 32.8 (C-15), 22.9 (C-16), 46.9 (C-17), 52.5 (C-18), 38.5 (C-19), 38.6 (C-20), 27.6 (C-21), 36.4 (C-22), 28.3 (C-23), 17.1 (C-24), 17.0 (C-25), 15.3 (C-26), 23.4 (C-27), 178.5 (C-28), 16.2 (C-29), 21.2 (C-29). These physical properties correspond to those of the UA reported in previous studies [9, 24, 25].

2.2. Ursolic acid gel

Ursolic acid was formulated into gels using two types of gelling agents, i.e., carbomer and HPMC. Carbomer is a synthetic polyacrylic acid resin copolymerized with about 0.75-2% polyalkyl sucrose. This is the reason why the dispersion of carbomer in the water should be protected against microbial growth. On the other hand, HPMC is a semi-synthetic gelling agent of cellulose derivatives that is resistant to phenol and stable at pH 3-11 [26]. In this study, HPMC formed a clearer UA gel than carbomer (Figure 2). This is in line with the previous study. In the accelerated stability test, diltiazem hydrochloride gel with HPMC as gelling agent showed better stability compared to that of using polyethylene oxide. Even after exposure to heat and humidity, there was no significant change in clarity and texture properties [27].

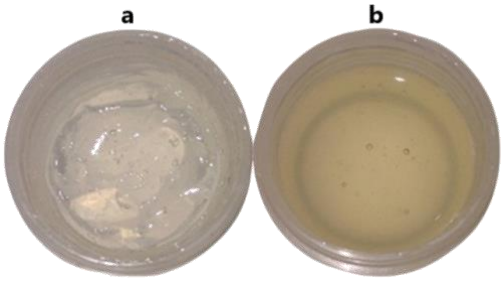

Figure 2. Ursolic acid gel with carbomer (a) and HPMC (b) as gelling agents

2.3. Stability test

Visual appearances of UA gels were evaluated for 90 days with an observation interval of 15 days. During which, no degradation was apparent in the two gels formulated using carbomer and HPMC. In other words, gel consistency, translucence, and odorless characteristic were maintained.

Table 1 and Figure 3 show the gels' viscosity in the stability testing. This physical property determines the flocculation rate. Flocculation itself might occur if the gel's viscosity increases too early. Based on Table 1 and Figure 3, carbomer was the gelling agent that produced more stable gels than HPMC.

Table 1. Viscosity of UA gels under stability testing conditions

Day of	Viscosity (cPs)*	
	Formula I**	**Formula II****
0	14843 ± 127	12698 ± 181
15	14408 ± 81	12404 ± 244
30	13776 ± 372	11807 ± 132
45	13298 ± 530	10447 ± 365
60	13048 ± 207	9838 ± 123
75	12759 ± 224	9614 ± 110
90	12554 ± 221	9064 ± 58

*Measurements were conducted using a viscometer (Brookfield type cone and plate; spindle number CP-41 at 27±2°C; 0.5 rpm).
**Data are expressed in mean ± SD with n = 3; Formula I: UA gel with carbomer as a gelling agent; Formula II: UA gel with HPMC as a gelling agent.

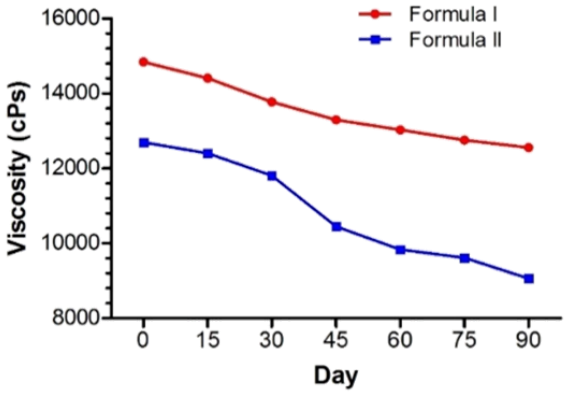

Figure 3. Viscosity profile of UA gels

Figure 4 shows the flow properties of the formulated UA gels. The two graphs indicate that the UA gels prepared using carbomer and HPMC as the gelling agents were both pseudoplastic. This characteristic means that an increase in the shear rate will be followed by a decrease in viscosity [28].

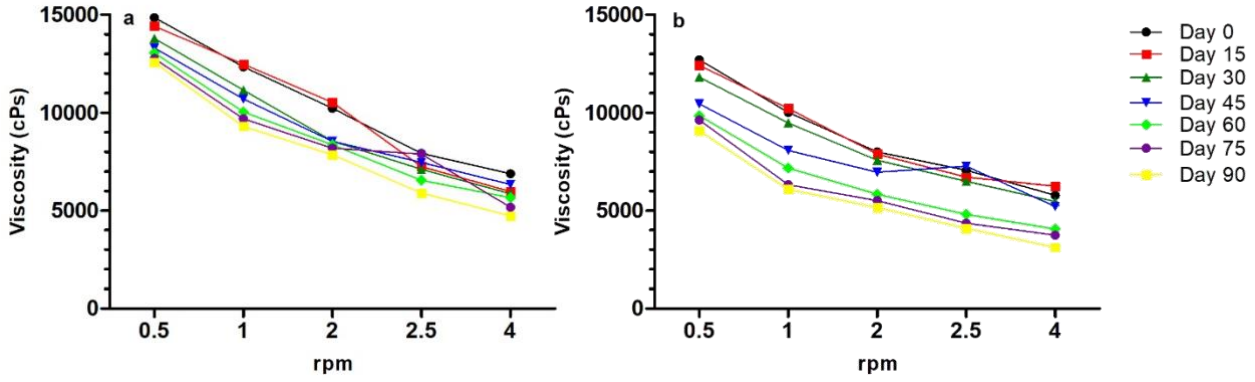

Figure 4. Flow properties of UA gel using carbomer (a) and HPMC (b) as gelling agents
Note: measurements were conducted using a viscometer (Brookfield type cone and plate; spindle number CP-41 at 27 ± 2°C; 0.5 rpm)

The pH levels of the UA gels are presented in Table 2. During the 90-day accelerated stability test, the pH levels of formula I changed from 4.6 ± 0.06 on Day 0 to 4.5 ± 0.04 on Day 90, while those of formula II were 5.8 ± 0.04 on Day 0 and then decreased to 5.7 ± 0.02 on Day 90. The decreasing trends over the 90 days of observation were insignificant, indicating the two formulas' ability to maintain the pH levels for 90 days

($P > 0.05$). The derived pH levels met the pH criterion of a topical preparation, hovering around the skin pH range, 4.0–7.0 [29]. Gels with too alkaline character can cause scaly skin, and too acidic gels will make the skin irritated. Therefore, UA gels with pH 4.5-5.8 are considered suitable for skin.

Table 2. pH of UA gels under stability testing conditions

Day of	pH	
	Formula I	**Formula II**
0	4.6 ± 0.06	5.8 ± 0.04
15	4.5 ± 0.03	5.7 ± 0.03
30	4.5 ± 0.03	5.7 ± 0.05
45	4.5 ± 0.05	5.7 ± 0.10
60	4.5 ± 0.04	5.7 ± 0.04
75	4.5 ± 0.03	5.7 ± 0.03
90	4.5 ± 0.04	5.7 ± 0.02

*Data are expressed in mean ± SD with n = 3; Formula I: UA gel with carbomer as a gelling agent; Formula II: UA gel with HPMC as a gelling agent.

2.4. Method validation

In the research, UA contents of the formulated gels were quantified using TLC-densitometry. This method was then validated based on five parameters: specificity, linearity, accuracy, precision, LOD and LOQ. First, based on the TLC chromatogram (Figure 5) and densitograms compared in Figure 6, the method was specific for UA because the compound was successfully separated without apparent impurities from other components of the gel dosage forms. TLC chromatograms of sample (Figure 5, tracks c and d) show purple bands under white light after derivatization with 5% sulfuric acid in methanol, with a position parallel to the UA standard.

Figure 5. TLC chromatogram of UA standard 20.24 ng/spot (a) and 60.72 ng/spot (b); gel containing UA 42.06 ng/spot (c) and 56.08 ng/spot (d)

The purity of the detected UA was also seen in the visible spectrum of the UA standard, which was superimposed on the sample's peak (Figure 7). The visible spectra of the UA standard and sample showed a similar pattern, each at a maximum wavelength of 536 nm. This maximum wavelength corresponds to the one used in a previous study, i.e., 530 nm [21] and 540 nm [30].

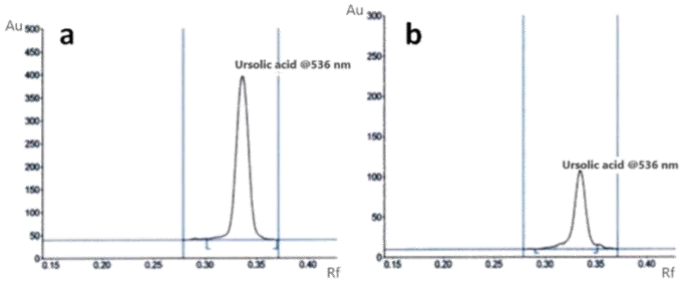

Figure 6. Densitograms comparing UA standard (a) with UA gel (b)

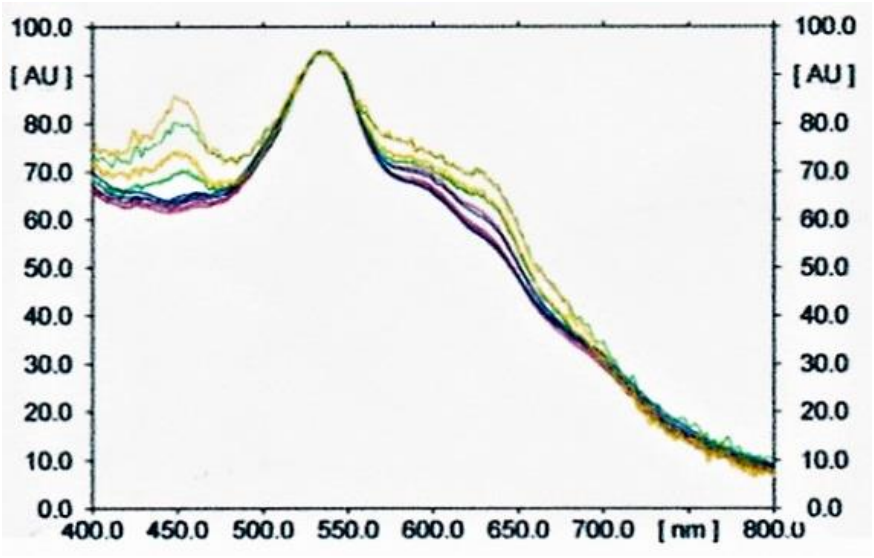

Figure 7. Overlay spectra of UA standard with the corresponding compound in the sample

Second, after plotting the peak areas (*y*-axis) against UA concentrations (*x*-axis), a good linear correlation ($r \geq 0.998$) was formed between the two for the UA in the range of 20–110 ng/spot (Figure 8). Previous research showed that UA has a good linear relationship (r=0.9986) over the concentration range 200–600 ng/spot with respect to peak area [21]. Our findings indicate that the developed TLC-densitometric method is capable of analyzing low UA concentrations in the gel dosage forms.

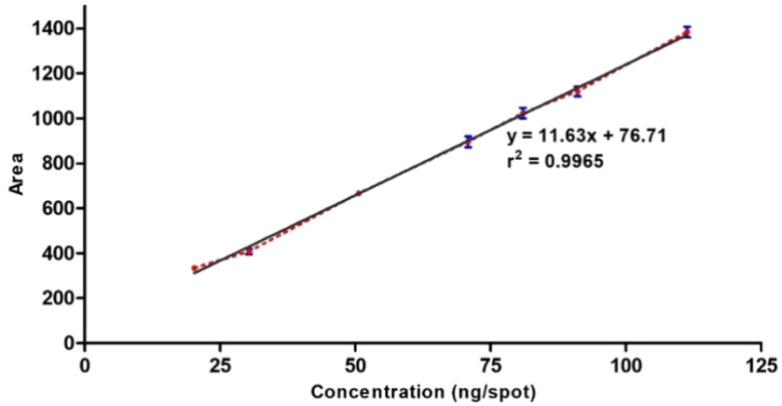

Figure 8. Calibration curve of UA

Third, Table 3 shows the accuracy testing results (as %recovery). Based on the various amounts of sample applied, the recovery was between 95.04 and 116.82%. Since the recoveries were in the range of 80-

120%, the proposed TLC method is therefore accurate [31]. Fourth, to determine the method's precision, the study used the relative standard deviation (RSD) for the data collected within one day (intraday) and between several days (interday). The RSD values of the intraday precision (1.24–1.96%) and interday precision (≤ 6.29%) indicated a precise method. With a different concentration of UA, 200-600 ng/spot [21] and 1000-3000 ng/spot [30], the HPTLC-densitometer in previous studies also had good intraday and interday precision (RSD ≤ 2%). Fifth, the last parameters of method validation are LOD and LOQ. The lowest UA level at which the method could detect (LOD) and quantify (LOQ) was, respectively, 4.55 ng/spot (LOD) and 15.17 ng/spot (LOQ). These LOD and LOQ values are lower than that found by Patel and Vyas [21] which is 10.02 ng/spot and 30.36 ng/spot, respectively and even much lower than that found by Jamal *et al*. [30] i.e. 561.85 ng/spot and 1702.58 ng/spot, respectively. The study results suggested that the developed TLC-densitometry was sensitive; thus it is sufficient to evaluate the UA content in the gel dosage forms.

Table 3. Accuracy test results of the gels' ursolic acid using TLC-densitometry

Applied (ng)	Found (ng, n = 3)	Recovery (%)	RSD (%)*
105	123.02	116.82	0.91
69	75.68	109.60	0.68
60	57.15	95.04	1.99

*RSD refers to the %relative standard deviation obtained from triplicate measurements.

3. CONCLUSION

The ursolic acid in the gel dosage forms is stable during the accelerated stability test that lasted for 90 days. In addition, based on the method validation results, the TLC-densitometry proposed in the study is specific, precise, and accurate and shows a good linearity; thus, it can be used to detect, separate, and quantify ursolic acid in a gel preparation.

4. MATERIALS AND METHODS

4.1. Chemicals and equipment

The study used pharmaceutical-grade chemicals, namely, carbomer 940, hydroxy propyl methyl cellulose (HPMC), methylparaben, propylparaben, NaOH, propylene glycol, ethanol, and aqua purificata. All analytical grade solvents were procured from Merck KGaA (Darmstadt, Germany). The TLC plates were pre-coated with silica gel 60 F_{254} and different silica gel types, which were obtained from Merck KGaA (Darmstadt, Germany). As for the standard compound (UA), it was obtained from Sigma (St. Louis, MO, USA). The equipment in this study consisted of a viscometer (cone/plate, Brookfield, AT 71362), pH meter (Schott Lab 850), climatic chamber (KBF 240), an analytical balance (Ohaus), water bath (Memmert), mortar and stamper, homogenizer (Multimix), exicator, and laboratory glassware.

4.2. UA extraction, isolation, and identification

The plant material used in this research was *Plantago major* leaves harvested in Tawangmangu, Province of Jawa Tengah, Indonesia. The plants had been authenticated by the Center of Information and Development of Traditional Medicine, University of Surabaya, Indonesia (No. 1101/D.T/XI/2013). UA was then isolated according to the method used in a previous study [3]. First, crude drugs were grounded into fine-sized powder (2 mm) before being macerated with methanol. Second, in a vacuum environment, the methanol extract was evaporated to produce a concentrated extract. Then, column chromatography was performed, where the extract was passed through a silica gel column to yield a pure compound. The physical data (visual appearance, melting point) and the results of the IR spectroscopy, ESI-MS, and nuclear magnetic resonance (^{1}H NMR, ^{13}C NMR) conducted on the isolated compound were analyzed

4.3. UA gel formulation

The UA gel was prepared with two types of gelling agents according to Sudjono, i.e., carbomer (formula I) and HPMC (formula II) [32]. The composition of the gel is shown in Table 4. Two grams of carbomer were dispersed on water and allowed to stand for 30 minutes, then stirred rapidly with a homogenizer. Sodium hydroxide solution was then added gradually while stirring until a transparent gel mass. Cold water was subsequently added and stirred until homogeneous. Next, UA was dissolved in ethanol, while methylparaben and propylparaben were dissolved in propylene glycol. The UA solution was

then added to the preservative solution. The mixture was then added to the gel base and stirred until homogeneous.

The gel with HPMC as a gelling agent was prepared as follows. Two grams of HPMC were dispersed on water and then allowed to stand for 30 minutes, stirred rapidly with a homogenizer. After the air bubbles were removed, cold water was added and stirred until homogeneous. Next, UA and methylparaben-propylparaben were dissolved in ethanol and propylene glycol, respectively. Next step, UA solution was added to the preservative solution and then to the gel base. Afterward, the mixture was stirred until homogenous.

4.4. pH measurement and physical stability test

To measure pH and examine the gel's physical stability, an accelerated stability study was conducted for three months with the following conditions: temperatures at 40 ± 2°C and relative humidity (RH) of 75 ± 5%. In addition to pH, the physical stability tested organoleptic properties (i.e., color, odor, and consistency) and determined viscosity and flow behavior. First, a viscometer (cone and plate, spindle number CP-41 at 27 ± 2°C and various shear rates) was used to measure the gel's viscosity. Measurements were conducted at the shear rate of 0.5 rpm. Then, flow properties were determined by plotting the shear rate used (0.5–4 rpm) *vs.* the viscosity.

Table 4. Composition of the ursolic acid gel

Materials	Composition (%w/w)	
	Formula I	Formula II
Ursolic acid	0.0075	0.0075
Carbomer	2	-
HPMC	-	2
Methylparaben	0.18	0.18
Propylparaben	0.02	0.02
1% NaOH	1	-
Propylene glycol	16	16
Ethanol	2	2
Aqua purificata to	100	100

4.5. Method validation

4.5.1. TLC instrumentation and condition

Chromatography used TLC silica gel 60 F_{254} plates sized 20x10 cm (Merck, Darmstadt, Germany). First, each standard solution and sample was spotted onto the plates (6 mm bands) using a Camag Linomat 5 sample applicator with the help of N_2 gas flow. The spotting began about 15 mm from the left edge with a 15 mm margin from the bottom edge and a 10 mm distance between the spots. Prior to development, the mobile phase consisting of toluene, ethyl acetate, and formic acid at the ratio of 8 to 2 to 0.1 was used to equilibrate in the Camag twin-through chamber (20×20 cm) for 20 minutes at room temperature. The plates were then developed (linear, ascending) with 30 ml of the mobile phase until it traveled a distance of 8 cm. Afterward, the plates were blown with warm air for drying and dipped in 5% sulfuric acid in methanol for derivatization. Before performing the densitometry, the plates were re-dried in a fume hood, placed in a preheated oven, and heated at 120°C for 7 minutes. Finally, the Camag TLC Scanner 4, controlled by winCATS software, was used to quantitatively analyze the data obtained at 536 nm, with a 4×0.3 mm slit, data resolution of 1 nm/step, and a scanning speed of 100 nm/s.

4.5.2. Calibration, linearity, LOD and LOQ

To create the calibration curves, UA (2.5 mg) was dissolved in 10 ml of methanol, creating a 250 µg/ml stock solution. This concentration was later diluted to 10 µg/ml. Afterward, the diluted solution was spotted onto the TLC plates at seven different volumes, i.e., 20, 30, 50, 70, 80, 90, and 110 ng/spot, and this procedure was conducted three times (triplicate). The linear regression between peak areas and UA concentration per spot was analyzed to produce a standard curve. Then, from the calibration curve, LOD and LOQ were calculated using the intercept (standard deviation, y-axis) and slope (mean, x-axis) of the regression line equation. LOD and LOQ are 3.3 and 10 times the standard deviation-mean ratio, respectively, as presented below.

$$LOD = 3.3\frac{SD}{m}; LOQ = 10\frac{SD}{m}$$

4.5.3. Method's specificity, accuracy, and precision

Five grams of the UA gel were sonicated using 10 ml of methanol for 45 minutes. The mixture was then centrifuged to separate the UA solution from the residue of the gel base. To determine the method's specificity, the spectrum of the UA standard was overlaid with the sample's corresponding peak (400–800 nm). The placebo method was used to assess the method's accuracy. It was performed by adding UA to a certain amount of gel with a range of 80–120%. Determination was conducted in three different concentrations, three replications each. Afterward, for the method's precision, the relative standard deviation (%RSD) was computed from the results of triplicate measurements. Here, a standard solution at 50 ng/spot was used to determine intraday precision (within one day) and interday precision (three days in a row).

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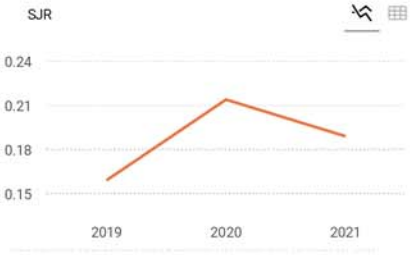

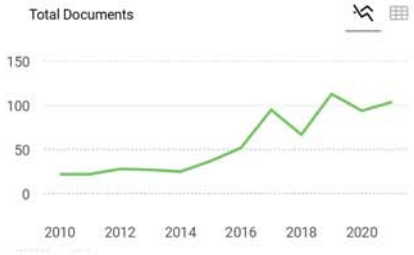

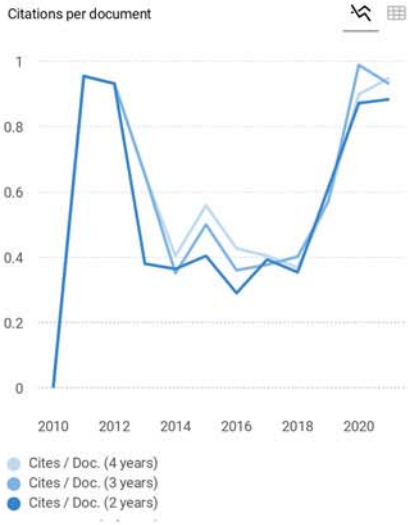

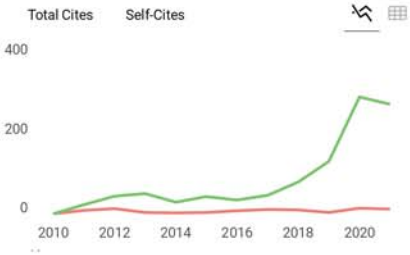

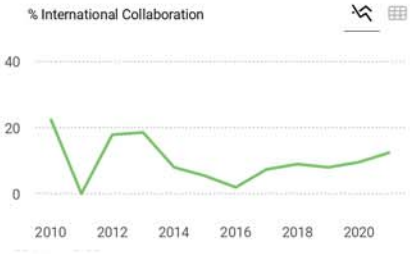

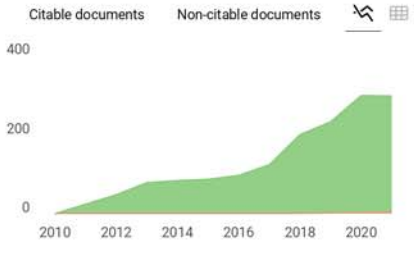

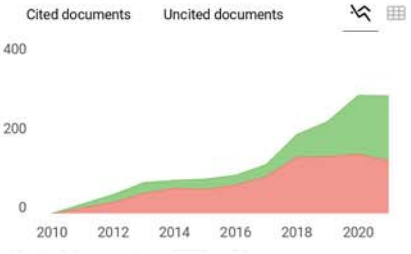

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