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RESEARCH ARTICLE

The colloidal gold nanoparticle-based lateral flow immunoassay for fast and simple detection of plant-derived doping agent, higenamine

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Abstract

Higenamine (HM), an alkaloid found in various plant species, is obtained when norcoclaurine synthase selectively condenses dopamine and 4-hydroxypheny lacetaldehyde to give (*S*)-higenamine ((*S*)-HM). The World Anti-doping Agency has listed HM as a prohibited agent in athletics. As a result, many commercial, academic, and regulatory bodies across the globe are invested in finding a rapid method for (*S*)-HM detection. In the current study, a lateral flow immunoassay (LFA) was developed in which the relevant biosensor was generated as a conjugate of the monoclonal antibody against (*S*)-HM (namely, MAb E8) and colloidal gold nanoparticles. The HM–γ-globulin conjugates and rabbit anti-mouse IgG antibodies were placed in the test and control zones, respectively. The free (*S*)-HM molecules in the samples and the immobilized HM–γ-globulin conjugates competitively reacted with the developed biosensor in the LFA. An inverse relationship existed between the biosensors' visible response, which was noted by the variation in the intensity of a pinkish spot in the test zone, and the content of the free (*S*)-HM. The limit of detection of the developed LFA was 156 ng/mL. Various validation methods confirmed that the LFA exhibited sufficient sensitivity, selectivity, repeatability, and reliability, making it ideal for (*S*)-HM detection in plant samples and plant-containing products. The developed system required only a small sample volume (20 μL) and a concise sample preparation time compared with conventional LFAs. Thus, the LFA reported in this study could serve as a rapid response kit for the detection of (*S*)-HM in plant samples.

KEYWORDS

colloidal gold nanoparticles, higenamine, immunochromatographic test strips, lateral flow immunoassay, monoclonal antibody

1 | INTRODUCTION

Higenamine (HM; 1-[(4-hydroxyphenyl)methyl]-1,2,3,4-tetrahydroiso quinoline-6,7-diol, Figure 1) is a benzyl tetrahydroisoquinoline alkaloid derived from various plant species, including *Aconitum carmichaelii* Debeaux. (Ranunculaceae), *Nandina domestica* Thunb., (Berberidaceae), *Nelumbo nucifera* Gaertn. (Nelumbonaceae), and *Asarum heterotrophies* F. Schmidt (Aristolochiaceae).[1,2] Given its chiral character, studies have revealed that the norcoclaurine synthase conformational selectively condenses dopamine and 4-hydroxyphenylacetaldehyde to form the specific enantiomer, (*S*)-HM.[3–5] From a pharmacological perspective, HM is a partial

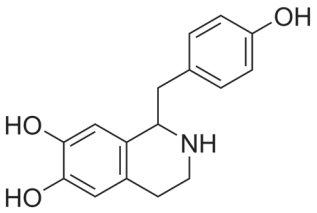

FIGURE 1 Chemical structure of higenamine (HM)

agonist of the beta-adrenergic receptor (β-AR)[6] that exhibits anti-analgesic,[1] anti-oxidative,[7] anti-inflammatory,[8] and antitumor properties.[9] Recently, HM was shown to improve the condition of the cerebral ischemia–reperfusion injury in vivo.[10] HM is commonly used in dietary supplements and as a performance enhancer, which has prompted the World Anti-doping Agency (WADA) to classify HM as a permanently banned substance under the "S3 group beta-2 agonist" listing as of January 1, 2017.[11] This ban has put considerable strain on anti-doping testing facilities as there is a high demand for rapid, accurate, reliable testing methods for the quantitation of HM in test samples.

The development of HM detection methods first started in 1987 and utilized high-performance liquid chromatography–ultraviolet (HPLC–UV) spectroscopy for quantitation purposes.[12] Even though the subsequent introduction of methods such as gas chromatography–mass spectrometry (GC–MS) combined with derivatization techniques[13] and ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS)[14] has resulted in the development of markedly improved testing protocols, these methods are not always convenient to implement due to the need for expensive reagents, cumbersome equipment, high organic solvent consumption levels, complex sample treatment and preparation protocols, and time-consuming analysis.

Immunoassays using monoclonal antibodies and their relevant fragments are gaining popularity for detecting natural compounds and phytochemicals[15] as these systems are extremely specific and can be applied for both quantitative and qualitative analyses. An anti-(*S*)-HM monoclonal antibody (MAb E8) was developed in conjugation with sensitive and reliable indirect competitive enzyme-linked immunosorbent assay (icELISA) for (*S*)-HM detection.[16] Despite its quantitative advantages, this icELISA-based method is plagued with limitations as the procedure is requiring multiple steps such as antigen coating, nonspecific protein blocking, primary antibody reaction, secondary antibody reaction, and substrate addition, all of which culminate in a total assay time of at least 5 h. Additionally, icELISA requires a specific microplate reader for signal detection. These points serve to make a powerful detection tool such as this almost inaccessible outside laboratory settings.

Herein, a lateral flow immunoassay (LFA) was developed for (*S*)-HM detection since LFAs have a long history of reliable usage and are renowned for their simplicity and fast immunoassay response for semi-quantitative or qualitative analyses. The ease of preparation, simple interpretation of the results, eco-friendliness of the process, rapid analysis, and cost-effectiveness make this method ideal for the detection of secondary metabolites in plants. To the best of our knowledge, this is the first reported use of LFA for (*S*)-HM detection. The goal of this study is to develop a colloidal gold-based LFA using our previously produced MAb E8 antibody for (*S*)-HM detection in plant samples. One notable merit of this method over the conventional icELISA technique is speed as the developed LFA method takes only 20 min to complete an analytical protocol, whereas several hours are required for icELISA. Moreover, the developed method is a variant of the microplate reader-free method, and the results can be easily interpreted via visual observation or simple photographic analysis using the appropriate software. Moreover, the developed strip test is in the preincubation format, meaning that there is no need to prepare the colloidal gold fiber pad prior to use. The application of the developed LFA for (*S*)-HM detection under real-world circumstances is discussed in the manuscript.

2 | EXPERIMENTAL

2.1 | Chemicals and reagents

(*S*)-Higenamine HBr (98%) and higenamine HBr (98%) were purchased from Toronto Research Chemicals (Toronto, Canada). *N*,*N*-Carbonyldiimidazole (CDI) and albumin from chicken egg white (OVA; 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). γ-Globulin from human serum (95%) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The colloidal gold nanoparticle suspension (0.0070 wt% with a mean size of 15 nm) was purchased from Tanaka Holding Co. (Kanagawa, Japan). Rabbit anti-mouse IgG pAb (ab6709) and goat anti-mouse IgG1 (HRP) (ab97240) antibodies were purchased from Abcam (Cambridge, MA, USA). All other reagents were purchased as analytical grade.

2.2 | Plant sample preparation

The plant samples were purchased from Uchida Wakanyaku Co. Ltd. (Tokyo, Japan). *N. domestica*-containing candy, that is, Tokiwa Nanten Nodo Ame (22 tablets/pouch; brown sugar and honey–plum flavor), were purchased from a Japanese drug store (Fukuoka, Japan). Four samples, that is, *N. domestica* (fruit and leaf), *Asarum siebodii* Miq. (root), and *Evodia rutaecarpa* (Juss.) Benth. (fruit), were milled, and the powders were passed through a 300-μm mesh sieve prior to their use in subsequent steps. Each powdered sample (30 g) was macerated in MeOH (300 mL) and left overnight at room temperature. The MeOH extracts of the plant samples were prepared by first filtering the mixture and removing the solvent under vacuum before reconstituting the sample in MeOH (10 mL). For *A. carmichaelii* roots (Bushi, Shuchi-Bushi, and Houbushi), the samples were handled using the same preparation method with one exception; here, the dry weights of the plant samples (50 g) were used. The respective candy samples

(1.6 g each) were simply dissolved in 5% MeOH (2.0 mL). All samples were diluted 20-fold in 5% MeOH before analysis.

2.3 | Preparation of the HM–protein conjugates

HM was conjugated to γ-globulin obtained from human serum and placed in the test zone of the immunochromatographic test strip. Additionally, HM–OVA conjugates were synthesized and used as the coating antigen for the icELISA, as described in a previous study.[16] The hydroxy groups of HM served as the reaction sites for carbamate-mediated protein conjugation. HM (3.2 mg) and CDI (3.1 mg) were mixed in anhydrous DMF (0.6 mL) and stirred at 30°C for 3 h to facilitate the formation of the reactive intermediate, which was then allowed to undergo conjugation with the human serum-derived γ-globulin (6.1 mg) that had been diluted in distilled water (2.4 mL), via the dropwise addition. The reaction progressed overnight at room temperature after which the unreacted compounds were removed via dialysis using 1 L of distilled water at 4°C with three daily water change procedures for 2 days. The HM–γ-globulin conjugates (4.1 mg) were obtained after the dialysate was lyophilized. The HM–OVA conjugates were prepared via the same protocol as that employed for preparing the HM–γ-globulin conjugates using HM (3.3 mg), CDI (4.1 mg), and OVA (6.5 mg). The HM–OVA conjugates (5.4 mg) were obtained after lyophilization.

2.4 | MAb E8–colloidal gold nanoparticle conjugation

The antibody–colloidal gold nanoparticle conjugates were used as the biosensors for the developed LFA and were prepared via a previously reported protocol with some modifications.[17] Briefly, the environment of the colloidal gold nanoparticles (1 mL) was adjusted to be weakly alkaline (i.e., pH = 9.0) using a potassium carbonate buffer solution (20 μL). Then, MAb E8 (50 μg) was mixed with the pH-adjusted colloidal gold suspension, and the mixture was left on an orbital shaker at 50 rpm for 10 min at room temperature. A mixture composed of 10% (w/v) BSA and 5% polyethylene glycol (PEG20000) in a 100-mM Tris buffer solution at pH 8.0 was added to the antibody–colloidal gold nanoparticle suspension to stabilize the resulting conjugates; the final concentrations of BSA and PEG20000 were 1.0% and 0.5%, respectively. The mixture was shaken using an orbital shaker at 50 rpm for 1 h at room temperature after which the conjugates were harvested via centrifugation at 7400 g for 30 min at 4°C. The pellets were re-dispersed in 1% (w/v) BSA in a 100-mM Tris buffer solution at pH 8.0 (1 mL) to remove the unwanted residue and subsequently centrifuged at 7400 g for 30 min at 4°C. The washed pellets were carefully suspended in a 1% (w/v) BSA solution (35 μL) and stored at 4°C for future use. The detection mixture for each test strip was prepared prior to use. Briefly, the suspension containing the MAb E8 conjugates (8 μL) was mixed with 10% (w/v) sucrose in distilled water (45 μL), 1% (v/v) Tween 20 in distilled water (21 μL), 1% (w/v) BSA in a 100-mM Tris–HCl buffer solution (pH 8.0, 32 μL), and distilled water (24 μL). This detection mixture (130 μL) was combined with the respective analyte (20 μL), namely, the (*S*)-HM standard, the cross-reactivity testing compounds (20 μg/mL), or the plant extracts. The analytes of interest were adjusted to obtain a final MeOH concentration of 5%. The analyte of interest and colloidal gold conjugates suspension were mixed in a 96-well plate using a plate shaker and incubated for 5 min to facilitate the antibody–antigen reaction. The prepared immunochromatographic test strips (the "sample pad" end) were then immersed into the wells.

2.5 | Preparation of the immunochromatographic test strips

The developed LFA test strip was composed of three main parts, namely, the sample pad, a nitrocellulose backing, and the adsorbent pad (Figure 2). Cellulose fiber-based paper was used to construct the sample and adsorbent pads. This paper was cut into 1.5 × 0.6 cm strips for both the sample and adsorbent pads. The nitrocellulose backing (Hi-Flow Plus 240, Millipore, Temecula, CA, USA) was cut into 5.4 × 0.6 cm strips. The capture zones on the nitrocellulose membrane, that is, the control and test zones, were clearly marked. Rabbit anti-mouse IgG pAb antibodies (1 mg/mL), which had been diluted with a 50-mM carbonate buffer containing 0.25% (w/v) sodium dodecyl sulfate (SDS), were applied to the control zone of the membrane (0.5 μL, which was equivalent to 0.5 μg). For the test zone, the HM–γ-globulin conjugates (2 mg/mL), which were diluted in a 50-mM carbonate buffer containing 0.25% (w/v) SDS, were applied to the membrane in 0.5-μL drops for three rounds of applications (which was equivalent to 3 μg); each time that the solution was applied to the test zone, the membrane was allowed to dry at room temperature prior to another round of applications. The control and test zones were spotted far from each other at a distance of 5 mm to ensure that there was no overlap. When the coated membrane was completely dry at room temperature, the membrane was soaked in 1% (w/v) BSA in PBS for 2 h. The membrane was then washed thrice with a phosphate-buffered saline solution containing 0.05% (v/v) Tween 20 (PBS-T) before being allowed to dry at room temperature. Next, the dried nitrocellulose strip was assembled for analysis with the sample pad on the bottom and the adsorbent pad on the top of the strip.

2.6 | The LFA procedure and interpretation

For analysis, the prepared strip (the "sample pad" end) was immersed in the 96-well plate containing a mixture of the detection solution (130 μL) and the analyte of interest (20 μL) for a 5-min incubation period. The control and test zones were monitored for the appearance of a pinkish tint after a reaction time of 15 min. If the control zone exhibited no color change, the test strip was marked as invalid, and the results were discarded. Photos of the results were taken using a phone camera and a scanner. Both the real test strips and the

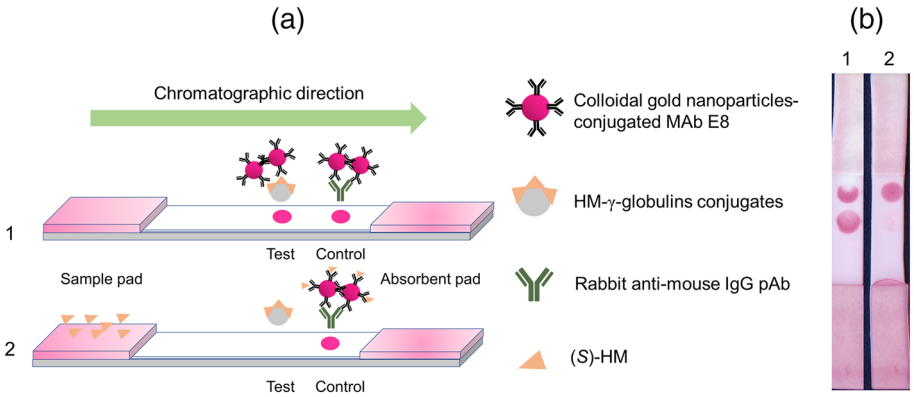

FIGURE 2 Lateral flow immunoassay (LFA) scheme illustration (a) with example strips (b). The detection mixture was flow in an anti-gravity direction from sample pad to adsorbent pad. (1) The scheme and result of LFA when using negative control (5% MeOH). (2) The scheme and result of LFA when the sample was 5000 ng/mL (*S*)-HM

photographs were visually monitored. Precise quantification of the color intensity of the test zone was conducted via the ImageJ software (National Institutes of Health, MD, USA) using the Region of Interest management tool. These results were plotted against the log of the (*S*)-HM concentration (ng/mL; Figure 3b).

2.7 | LFA quantitative method validation

The performance of the developed LFA was evaluated by determining the assay's sensitivity, repeatability, and cross-reactivity. The assay's sensitivity was tested using a series of (*S*)-HM dilutions ranging from 4.88 to 5000 ng/mL. The limit of detection (LOD) was marked as the highest concentration of (*S*)-HM that produced a color change in the test zone. The repeatability of the LFA was confirmed using the LOD of the intraday and interday assay results. The intraday assay was done by repeating the LFA on the same day (n = 3) after which the semi-quantitation range and LOD were compared for each run. The variations, which were expressed as the coefficients of variation percentage (CV%), were calculated using the test zone's intensity value obtained from the ImageJ software. For the interday assay, the LFA was performed on different days (n = 3), and the analysis was conducted in the same manner as that used for the intraday assay.

The cross-reactivity test is necessary for antibody-based assays since it is a direct indication of the assay's selectivity. Here, 27 natural compounds were investigated using the developed LFA at a screening concentration of 20 μg/mL. The compounds that exhibited positive results were subjected to further analysis by performing the LFA at various concentrations. The cross-reactivity (%) was determined using the LOD of both (*S*)-HM and the compound of interest:

$$\mathbf{CR}\text{s}\,(\%) = \frac{\text{LOD of}\,(S)-\text{HM}}{\text{LOD of cross}-\text{reacted compounds}} \times 100.$$

2.8 | Application of the developed LFA

The developed LFA was applied for (*S*)-HM detection in plant samples and candy containing extracts isolated from *N. domestica*. Since the assay was based on competitive inhibition of the enzyme's active site, an intense positive (+) result was noted as the absence of a pink color (relative to the results obtained in the 5% MeOH sample) in the test zone, whereas a weak positive result (±) was noted as a faint pink color. The negative result (–) was noted as the presence of a pink spot as intense as that observed for the control sample (5% MeOH). The working concentration of the plant or candy samples was a 20-fold dilution because it contained the final MeOH concentration of 5%. The results obtained from the reported LFA were compared with the results of the icELISA and HPLC–UV spectroscopic analysis that followed a previously described protocol.[16]

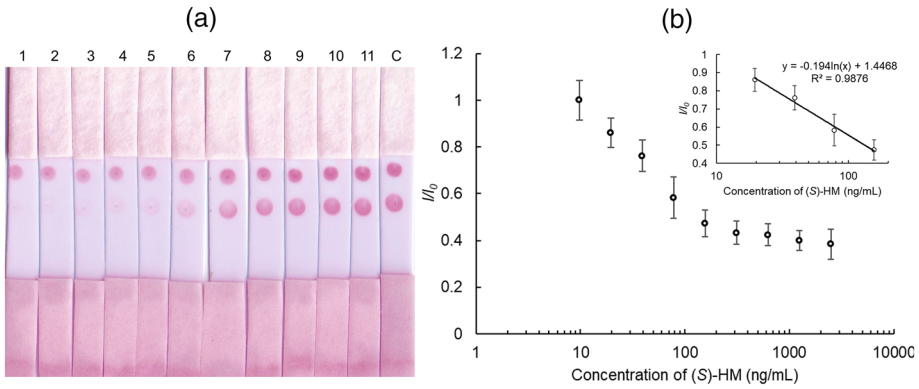

FIGURE 3 Lateral flow immunoassay (LFA) using (*S*)-HM standard. (a) The results were obtained from LFA using a set of (*S*)-HM concentration, which was twofold serially diluted from 5000 ng/mL (Lane 1) to 4.88 ng/mL (Lane 11) and the negative control (5% MeOH; Lane C). The error bars were the S.D. of the I/I_0 in each concentration obtained from interday assay. The inset picture was the semi-quantitative concentration curve

3 | RESULTS AND DISCUSSION

3.1 | The developed LFA preparation

Since WADA listed HM as a permanently banned performance enhancer,[11] numerous sports drinks and nutrition supplements have excluded it from their formulations. However, HM is a secondary metabolite in many plants. Intake of these plants leads to detectable HM in bodily secretions. Given these circumstances, a fast, effective method for screening the HM content in plant samples is needed. In this study, a simple, rapid assay was developed for (*S*)-HM detection in plant samples based on a competitive immunoassay using MAb E8 antibodies known to exhibit high specificity and sensitivity for (*S*)-HM.[16]

The LFA was developed by preparing test strips with nitrocellulose backing that could be immersed in 1% (w/v) BSA solution; this immobilized the reagents and reduced the nonspecific adsorption of the unwanted protein. A PBS-T solution was used for washing the prepared strips as it kept the membrane easy to be hydrated. After the detection solution and the analyte of interest were mixed in the well-plate, the "sample pad" end of the strip was immersed into the preincubated mixture and left to stand, thereby allowing the detection solution to flow slowly into the strip against the force of gravity via capillary motion. Sucrose was used to reduce the flow rate of the detection solution in our system, allowing for the optimal flow rate to be achieved. Since there was no fiber pad preparation step as is typical for conventional lateral flow assays,[15] the strip preparation time was reduced by at least 2 h, facilitating faster, easier analysis.

The MAb E8 antibody was randomly captured on the gold nanoparticle's surface via ionic and hydrophobic interactions. As illustrated in Figure 2a1, the MAb E8–colloidal gold nanoparticle conjugates interacted with the immobilized HM–γ-globulin conjugates in the test zone, whereas the anti-mouse IgG antibody reacted to the Fc region of the MAb E8–colloidal gold nanoparticle conjugates in the control zone in the absence of the (*S*)-HM analyte. This phenomenon caused a pinkish color in both the test and control zones. In the presence of (*S*)-HM, which was recognized as a positive result, the free (*S*)-HM molecules competitively bound to the MAb E8–colloidal gold nanoparticle conjugates, and the remaining MAb E8–colloidal gold nanoparticle conjugates were bound to the immobilized HM–γ-globulin conjugates, thereby causing a shift in the color intensity noted in the test zone when compared with the results seen in the control (5% MeOH; Figure 2a2). A higher (*S*)-HM content in the sample resulted in a weaker color transition (i.e., the pink color was faint) in the test zone. One key advantage was that the sample volume required for our LFA was exceptionally low (20 μL) compared with that required for conventional LFA[18] (400 μL) or icELISA[16] (50 μL) methods. This meant that our method was suitable for analyzing trace amounts of the analyte.

3.2 | Sensitivity of the LFA

Sensitivity is a crucial performance-indicating factor as it is vital for detecting even trace amounts of (*S*)-HM to prevent the misuse of the products containing the prohibited compound. Here, a set of (*S*)-HM standards of various concentrations (i.e., 4.88–5000 ng/mL) and the negative control (5% MeOH) were applied to the LFA (Figure 3a). Pink spots were observed in the control zone due to the presence of the rabbit anti-mouse IgG pAb antibodies, and the pink color of the test zone was due to the presence of the HM–γ-globulin conjugates of the negative control (5% MeOH) strip (Figure 3a; Lane C). Lanes 1–11 exhibited an intense pink color in the test zone when the concentration of the free (*S*)-HM was low. The pink spot disappeared when 313 ng/mL was used for LFA. Thus, the LOD of the developed LFA was 156 ng/mL, as determined via visualization. Regarding the sensitivity, there were many detection methods developed for higenamine detection. Our developed LFA shows higher sensitivity than developed HPLC–UV[16] (LOD of 0.207 μg/mL). Highly sensitive methods for higenamine detection have been developed (GC–MS combined with derivatization; LOD of 1.52 ng/mL,[13] UHPLC–MS/MS; LOD of 1 ng/mL,[14] and icELISA; LOD of 4.41 ng/mL[16]). However, our developed LFA was sensitive enough for (*S*)-higenamine in plant samples. The pictures of the strips were subjected to further analysis using ImageJ software. Here, we plotted the I/I_0 against the log of the concentration of (*S*)-HM (ng/mL), where I was the intensity obtained from the test zone at the individual (*S*)-HM concentration levels and I_0 was the intensity obtained from the test zone of the negative control (5% MeOH). We observed that the concentration between 19 and 156 ng/mL exhibited a correlation (Figure 3b); thus, this range (semi-quantitation range) could be used for estimating the (*S*)-HM concentration in this developed LFA.

3.3 | Repeatability of the LFA

The repeatability of the assay was confirmed by comparing the LOD of the intraday and interday assays. The results obtained via visualization revealed that the LOD remained the same at 156 ng/mL (Figure 4). Further investigations were conducted to determine the similarity of the I/I_0 obtained for each tested concentration when the LFA was performed on the same day (i.e., intraday analysis, $n = 3$) and on different days (i.e., interday analysis, $n = 3$). Since excessively high or low concentrations of (*S*)-HM could not sufficiently describe the repeatability of the assays, the coefficient of variation percentage (CV %) obtained from the concentrations within the semi-quantitation range was used as an indicator of precision. Table 1 shows that the highest variations in the intraday and interday assays were 9.96% and 14.93%, respectively, indicating that the developed LFA's repeatability was acceptable.

3.4 | Cross-reactivity of the MAb E8–gold nanoparticle conjugates

MAb E8 antibodies were employed in the developed LFA and were characterized in previous studies.[16] However, we noted that the cross-reactivity of the assay could be altered when the MAb E8 was

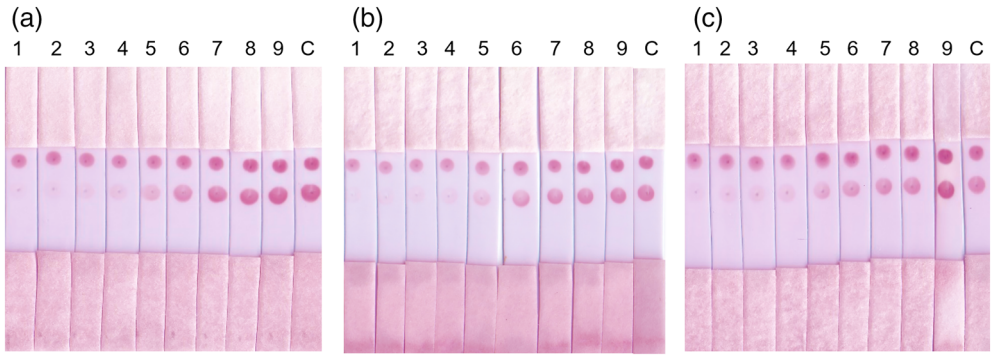

FIGURE 4 Repeatability of the lateral flow immunoassay (LFA). The results were obtained from LFA using a set of (*S*)-HM concentration that was twofold serially diluted from 2500 ng/mL (Lane 1) to 10 ng/mL (Lane 9) and the negative control (5% MeOH; lane C). Each sets of strips (a–c) were prepared in the different day

TABLE 1 Repeatability testing of the LFA indicated by the CV (%) obtained from semi-quantitation range of intraday and interday assay

	CV (%)[a]	
	Intraassay	Interday
(*S*)-HM concentration (ng/mL)	(*n* = 3)	(*n* = 3)
156	9.96	11.96
78	9.92	14.93
39	3.87	8.84
19	2.34	7.41

Note: The coefficient of variation (CV) was calculated using the following equation: CV = standard deviation (*SD*)/mean × 100.
Abbreviations: HM, higenamine; LFA, lateral flow immunoassay.
[a]The numerical value used for calculation was obtained from ImageJ; the I/I_0 of the test zone of the individual concentration was taken into an account.

attached to the gold nanoparticle. Thus, we applied 27 different natural compounds as LFA samples, the names and structures are provided in the Table S1 and Figure S1. As shown in Figure 5, the pink spots appeared in the test zone for all LFA strips except (*S*)-HM (Lane 1) and norlaudanosoline (Lane 9) when the concentration for screening was 20 μg/mL. Interestingly, the compounds that had previously exhibited the low cross-reactivity values, as determined in previous studies,[16] (namely, berberine and tetrahydrobenzylisoquinoline) exhibited no cross-reactivity in our developed LFA. There was the concern that a false positive might be observed when using this developed LFA with plant samples containing extremely high berberine and/or tetrahydrobenzylisoquinoline contents. The cross-reactivity of norlaudanosoline was further investigated by comparing the LOD of (*S*)-HM and that of norlaudanosoline. The results (Figure S2) showed that the LOD of norlaudanosoline was 78 ng/mL. The calculated cross-reactivity percentage was 200%, which was very similar to the results obtained using icELISA (223.04%). Even though high cross-reactivity was observed for norlaudanosoline, this compound is not commonly derived from plants; therefore, there is no threat of chemical interference during (*S*)-HM detection in plant samples.

3.5 | LFA application for (*S*)-HM detection in plant samples

The main purpose of the developed LFA was the accurate detection of (*S*)-HM in complex plant samples. Thus, the assay was subjected to sample matrices obtained from seven different plants and two candy samples derived from *Nandina dometica* extracts. The performance of the assay was determined by comparing the results obtained for the quantification of (*S*)-HM in the plant samples using several methods, namely, the reported LFA, icELISA, and HPLC–UV analysis (Table 2). The icELISA and HPLC–UV analyses were conducted using extracts that had been subjected to a 20-fold dilution procedure as the working concentration to obtain a final MeOH concentration of 5%. In this study, the *N. dometica*-containing candies were used as samples. The candies were generally used as an option for sore throat remedies in Japan. Consuming of these products may lead to detectable higenamine in urine, which can cause a conflict in a sport game. The solution prepared from the *N. dometica*-containing candy samples was also diluted 20-fold even though the final concentration of MeOH was already 5% in these samples; this was done because the extremely high sugar content affected the results of both the icELISA and LFA (data not shown). As shown in Table 2, the results of all three analytical methods were consonant with each other. The (*S*)-HM

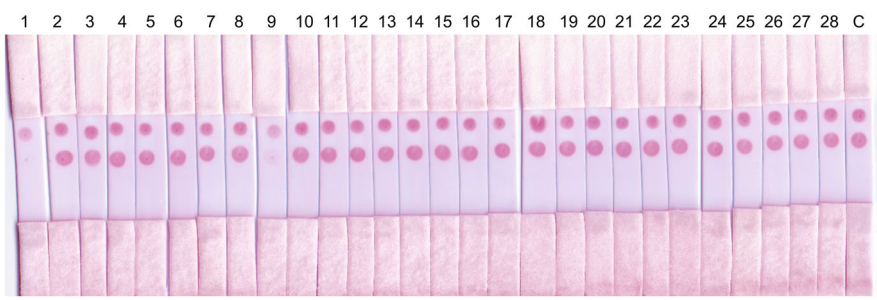

FIGURE 5 Cross-reactivity of lateral flow immunoassay (LFA). (*S*)-HM (Lane 1), 27 different naturals compounds (Lanes 2–28; 20 μg/mL), and negative control (5% MeOH; Lane C) were tested with developed LFA. The names and structures of these compounds were provided in Table S1 and Figure S1). The cross-reacted compound was norlaudanosoline (Lane 9)

TABLE 2 Comparative analysis of (*S*)-HM concentration in plants and plant-containing products using LFA, MAb E8 based-icELISA, and HPLC–UV

Sample	LFA[a]	(*S*)-HM concentration (μg/mL)[b]	
		icELISA	HPLC–UV
Plant samples			
Nandina domestica (fruit; Lane 1)	+	1.94 ± 0.06	2.22 ± 0.15
N. domestica (leaf; Lane 2)	+	8.57 ± 0.30	5.44 ± 0.83
Evodia rutaecarpa (fruit; Lane 3)	+	0.97 ± 0.11	1.40 ± 0.09
Asarum siebodii (root; Lane 4)	+	4.80 ± 0.38	3.56 ± 0.27
Aconitum carmichaelii (Houbushi, Uchida; Lane 5)	+	0.40 ± 0.02	0.36 ± 0.02
A. carmichaelii (Bushi, Uchida; Lane 6)	±	$0.09 \pm 2.60 \times 10^{-3}$	ND
A. carmichaelii (Shuchi-Bushi, Uchida; Lane 7)	+	1.07 ± 0.05	1.55 ± 0.01
***N. domestica* containing candy**			
Nanten nodo ame (brown sugar flavored; Lane 8)	±	0.22 ± 0.02	0.17 ± 0.01
Nanten nodo ame (honey–plum flavored; Lane 9)	±	$0.14 \pm 7.33 \times 10^{-3}$	0.20 ± 0.01

Abbreviations: HM, higenamine; HPLC–UV, high-performance liquid chromatography–ultraviolet; icELISA, indirect competitive enzyme-linked immunosorbent assay; LFA, lateral flow immunoassay; ND, not detected.

[a]Positive result (+) was the sample that causes no spot on the test zone and developed the pinkish spot on the control zone, weak positive result (±) was the sample that causes weak pinkish spot on test zone with present of pinkish spot on control zone, and negative result (–) was obtained from the sample which cause strong pinkish color on test and control zones.

[b]Concentration of (*S*)-HM in each sample was determined using the working concentration of LFA, which was 20-fold dilution of the sample extracts.

content in many samples represented the (*S*)-HM at various concentrations and in complex sample matrices. The (*S*)-HM concentrations in the samples that were higher than the LOD of LFA were positive when the developed LFA was used (Figure 6). The intensity of the color change associated with the (*S*)-HM standards and the plant samples were also compared. As shown in Figure 6, the intensity of the color change observed in the *A. carmichaelii* (Bushi, Uchida; Lane 6), Nanten nodo ame (Brown sugar flavor; Lane 8), and Nanten nodo ame (Honey–plum flavor; Lane 9) samples were comparable with the intensity observed for the (*S*)-HM standards that were 78 (Figure 3; Lane 7), 156 (Figure 3; Lane 6), and 156 ng/mL, respectively. This showed that the developed LFA was suitable for estimation of (*S*)-HM concentration in various plant samples.

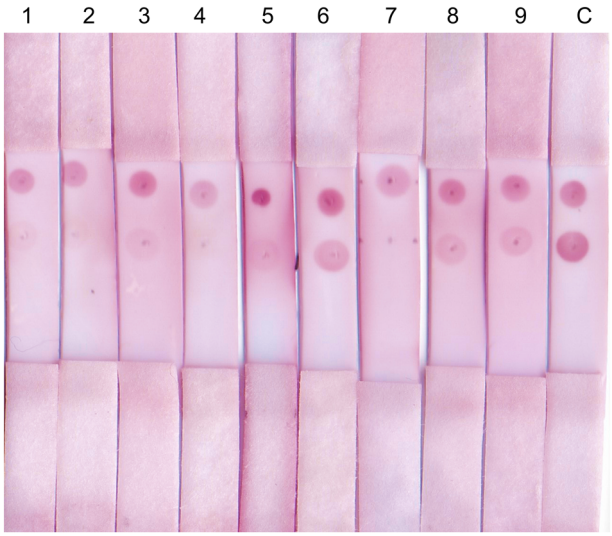

FIGURE 6 Lateral flow immunoassay (LFA) application. The LFA was used for (*S*)-HM detection in various plants. The positive results were shown when using 20-fold dilution extracts of *Nandina domestica* (fruit; Lane 1), *Nandina domestica* (leaf; Lane 2), *Evodia rutaecarpa* (fruit; Lane 3), *Asarum siebodii* (root; Lane 4), *Aconitum carmichaelii* (Houbushi, Uchida; Lane 5), and *Aconitum carmichaelii* (Shuchi-Bushi, Uchida; Lane 7). The weak positive results were shown when using 20-fold dilution extracts of *Aconitum carmichaelii* (Bushi, Uchida; lane 6), Nanten nodo ame (Brown sugar flavored; lane 8), and Nanten nodo ame (honey–plum flavored; Lane 9). The Lane C was negative control (5% MeOH)

4 | CONCLUSION

The LFA developed in this study was designed for the detection of (*S*)-HM in plant samples and phytochemical extracts. The principle of the assay features the competitive binding of MAb E8–gold nanoparticle conjugates to immobilized HM on test strips and free (*S*)-HM in the respective samples. Various validation methods were employed to establish the sensitivity, repeatability, and selectivity of the LFA, and the assay was applied to real-world plant samples and plant extracts to determine its performance and accuracy. Since only a small sample volume (20 μL) was required for analysis, this reported LFA is ideal in cases with limited sample volumes. Moreover, the employment of a preincubation strip format facilitated easier preparation and eliminated the tedious, time-consuming fiber pad preparation step necessary in conventional LFAs. To the best of our knowledge, the reported LFA is the first of its kind.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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