Sequential injection-chemiluminescence evaluation of stigmasterol glucoside and luteolin via green synthesis of silver nanoparticles using biomass of *Plectranthus asirensis*

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**ABSTRACT**

The present study focused on the phytochemical chromatographic isolation of two chemical constituents, sterol and flavonoid (stigmasterol glucoside and luteolin) from *Plectranthus asirensis*. A novel, eco-friendly, and cost benefit, ultrasensitive sequential injection analysis chemiluminescence (SIA-CL) approach based on the enhanced catalytic activity of silver nanoparticles (AgNPs) was suggested to evaluate the isolated compounds. Silver nanoparticles were synthesized using the biomass of an ethanolic extract of aerial parts of *P. asirensis* as a reducing agent. Spectroscopic and microscopic characterization of the prepared AgNPs were performed, including UV-Vis spectrometry, XRD and FT-IR as well as TEM and SEM. UV-spectroscopic method detected the formed AgNPs at an absorbance wavelength of 420 nm. Furthermore, antioxidant activity of the isolated compounds was also determined using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity protocol.

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**1. Introduction**

The genus *Plectranthus* L.’ He’r is represented by 300 species growing worldwide and is well known for its economic and medical importance (1). Several *Plectranthus* species are rich sources of essential oils with interesting biological activities, which are reportedly being used in several traditional as well as modern medicines (2,3). Also, various useful applications in the field of pharmaceutical and cosmetic industries have been reported in the literature for these essential oils (4). In folk medicine, many plant species of *Plectranthus* were used for the treatment of various diseases such as fever, digestive problems, skin diseases, respiratory problems, genitourinary problems, muscular skeletal conditions and various acute infections (5). The pharmacological properties reported for the genus include; antibacterial (6), anti-fungal (7), antiplasmodial (8), insecticidal (9), antioxidant, anti-inflammatory (10) and anticancer effects (11,8). Furthermore, extensive phytochemical investigation on different species of *Plectranthus* revealed that diterpenoids are the main chemical constituents of the genus *Plectranthus* besides the essential oils (12,2). Other constituents found are triterpenoids (13), steroids (14), phenolics (15), flavonoids (16,17) and fatty acids (2,4). In Saudi Arabia, genus *Plectranthus* is comprised of nine naturally growing aromatic species.
flowering plant species, which are often locally used in traditional medicinal practices, as ornamental and perfume products (18). *Plectranthus asirensis* J.R.I. Wood, a strongly aromatic plant, is found in tropical and non-tropical southwest Saudi Arabia and Yemen (19). Tradition *P. asirensis* is used in Saudi folk medicine for the prevention of various skin infections such as itching, diaper rashes and as an antiseptic for wound dressing (20). The thorough literature survey revealed that *P. asirensis* has not been subjected, previously, to any phytochemical or biological studies except for two reports that investigated the GC-MS composition (21) and the antimicrobial activity of leaves extracts in Saudi Arabia (22). Therefore, it was of interest to carry out a detailed phytochemical study of the plant with the aim to isolate biologically active constituents; and to quantitatively estimate the major biomarkers in different extracts.

Nanoparticles, relative to bulk materials, displayed improved features in their size, morphology and distribution. They are widely employed in various scientific fields (23). Various physical and chemical protocols have been exploited in the preparation of different types of nanoparticles. However, these methodologies still expensive and include the usage of hazardous and toxic chemicals, which may induce possible environmental and biological risks. Therefore, it is very important to increase awareness toward employing the green chemistry and biological processes to develop simple, cost-effective and eco-friendly procedures (24). Recently, the use of biological agents and plant extracts in the biosynthesis of nanoparticles and their applications holds an immense potential. Among metallic nanomaterials, silver nanoparticles (AgNPs), which possess unique physicochemical properties and exhibit good antimicrobial and medical benefits (25). AgNPs have a high aspect ratio regardless of their synthesis process, which reveals their surface characters such as solubility and stability. These characters are essential for AgNPs applications such as antimicrobial activity (26), catalysis (27), drug analysis (28), optical imaging (29) and electronics (30). AgNPs can be synthesized via chemical or green approaches. There are various chemical methods to obtain AgNPs such as photochemical reactions, thermal decomposition, electrochemical, and microwave-assisted. Those methods are usually having hazardous chemicals and wastes (31,32). Recently, green chemistry and biosynthetic approaches have grown to obtain AgNPs (33). These biomethods employ bacteria or fungi (34), marine algae (35), yeasts (36) or different alcoholic or aqueous plant extracts (37).

Although separation techniques provide a quick PC controlled and ultrasensitive detection of chemical compounds, they still possess many drawbacks such as high cost, consumption of large amounts of organic solvents and the need for high operator skills. Also, these techniques displayed some errors in analysis as well as they require environmental safeguard to minimize toxicity. In spectrophotometric methods such as spectrophotometry, a faulty device design with the light stray as well as other factors may affect the spectra measurement accuracy and hence influence the range of linear detection (38). Recently, the chemiluminescence (CL) technique as a quantitative detection method gained much opportunity and attention, due to its high sensitivity merits, (wider linear range and low background signals) (39).

The present study aims to isolate and confirm potentially and medicinally active sterol and flavonoid compounds (stigmasterol glucoside and luteolin) from *P. asirensis*. Furthermore, a novel, eco-friendly, cost benefit, ultrasensitive sequential injection analysis chemiluminescence (SIA-CL) system based on the enhanced catalytic activity of AgNPs was developed for quantitative evaluation of the isolated stigmasterol glucoside and luteolin. The antioxidant activity of the isolated compounds was studied using 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity protocol.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical grade and no further purification was required. Luminol (98.0%), potassium ferricyanide (99.0%), hydrochloric acid (36%) silver nitrate (99.0%) and ethanol (99.8%) were purchased from Sigma-Aldrich, (Hamburg, Germany). Sodium hydroxide (98.0%) and sodium dodecyl sulfate (SDS) were supplied by WINLAB, (East Midland, UK). Samples of (stigmasterol glucoside and luteolin) were isolated from an ethyl acetate fraction of *P. asirensis* plants.

2.2. Apparatus

IR spectra were recorded on Shimadzu Infrared-400 spectrophotometer (Shimadzu, Kyoto, Japan). ESI-MS spectra were recorded on a Finnigan MAT TSQ-7000 triple stage quadruple mass spectrometer (ThermoFinnigan, Bremen, Germany). HR-ESI-MS spectra were acquired using an LTQ Orbitrap mass spectrometer (Thermo Fisher, Waltham, MA, USA).\(^{1}\)H and \(^{13}\)C NMR spectra were measured using a Bruker DRX 700 spectrometers (Bruker, Rheinstetten, Germany). Silica gel 60 (0.04–0.063 mm mesh; 500 g; Merck, Darmstadt, Germany) was used to perform Vacuum liquid chromatography and column chromatography. SiO\(_2\) F\(_{254}\) aluminum precoated sheets (Merck, Darmstadt, Germany) were used.
for TLC analyses. CL detection was obtained by FIAlab-3500 instrument (Bellevue, Washington, USA). All experiments were computer controlled, model OPTIPLEX790 (DELL, China), to ensure precise timing of pump and valve movements. A digital balance, Lipra model GR-202 (AND, Japan), was used throughout the experiment. HANNA pH-meter (Cluj, Romania) was used for pH adjustment. UV-visible spectrophotometer model Uv-spectropec 2100° pro (Biochrom, England), with matched quartz cells (1 cm) was employed for characterization of the synthesized AgNPs. A transmission electron microscope (TEM) model JEM-1400 plus (JEOL, USA) and a scanning electron microscope (SEM) model JSM-7610F (JEOL, USA) were employed to ensure and detect the formation of the nanoparticles. Distilled water system model GFL Water Distillation Unit 2004 (CARL STUART GROUP, USA) were employed to ensure and detect the formation of the nanoparticles. Distilled water system model GFL Water Distillation Unit 2004 (CARL STUART GROUP, USA) were employed to ensure and detect the formation of the nanoparticles.

### 2.3. Plant materials

The aerial parts of *P. asirensis* were collected during (March 2013) from Abha in the south of Saudi Arabia at an altitude of ca. 2270 m above the sea level. Prof. Dr. Mohamed Yousef, pharmacognosy department, College of pharmacy, King Saud University, identified and authenticated the plant materials. A voucher specimen (PA-2567) has been preserved in the departmental herbarium.

### 2.4. Extraction and isolation

The powdered and air-dried aerial parts (1000 g) of *P. asirensis* were macerated in methanol (3 × 3 L, each) using soxhlet apparatus for 24 h with constant shaking at ambient temperature. All three extracts were filtered, centrifuged, combined and concentrated under reduced pressure to obtain a dark brown residue (20.5 g). The later was diluted with distilled water (150 mL) and the resulting suspension was sequentially partitioned between *n*-hexane (3 × 500 mL), ethyl acetate (3 × 500 mL), and *n*-butanol (3 × 500 mL) in separating funnels. Each fraction was individually concentrated to yield *n*-hexane (6.2 g), EtOAc (5.8 g), *n*-BuOH (2.4 g), and aqueous (5.4 g) fractions. Based on TLC profile, EtOAc (5.8 g) fraction was found rich in chemical constituents. The EtOAc (4.6 g) was taken up for liquid chromatography under vacuum using *n*-hexane: EtOAc gradient to produce four sub-fractions: PA-1 to PA-4. Sub-fraction PA-2 (55 mg) was subjected to adsorption column chromatography using a 230–400 mesh SiO2 (1:20 ratio) and eluted with *n*-hexane: EtOAc gradient, which resulted in isolation of milky white compound 1. The isolated compound 1 was purified using preparative TLC (SiO2 G plates; *n*-hexane: EtOAc: 9:1), which was further crystallized with methanol to give a white amorphous solid (60 mg) and was identified as stigmasterol glucoside on the basis of spectral analysis (40). Similarly, sub-fraction PA-3 that was eluted in *n*-hexane: EtOAc (20:80) gradient, compound 2 was isolated as a yellow residue. The above residue was further purified with chloroform to get a crude compound and repetitive crystallization of crude compound with methanol resulted in pure compound as yellow powder (56 mg), which was identified as luteolin in comparison with reported spectral data (41).

### 2.5. Spectral data

**Compound 1:** White amorphous solid; positive to Liebermann-Burchard test; *Rf*, 0.54; mp 282–286 °C; IR *ν*~max~ cm⁻¹: 3402.44 (OH), 2830.65 (CH), 1121.24 (C–O), 1640.21 (C = C). ¹H NMR (500 MHz, CDCl₃) 1.70 & 1.52 (2H, m, H-1), 2.31 & 1.81 (2H, m, H-2), 4.21 (1H, m, H-3), 2.27 & 1.48 (1H, m, H-4), 5.37 (1H, t, *J* = 4.5 Hz, H-6), 2.07 & 1.57 (2H, m, H-7), 1.46 (1H, m, H-8), 1. 15 (1H, m, H-9), 1.57 & 1.29 (2H, m, H-11), 1.47 & 1.23 (1H, m, H-12), 0.81 (1H, m, H-14), 1.87 & 1.26 (2H, m, H-15), 1.72 & 1.52 (2H, m, H-16), 1.19 (1H, m, H-17), 0.71 (3H, s, H-18), 1.01 (3H, s, H-19), 2.12 (1H, m, H-20), 0.95 (3H, d, H = 7.2 Hz, H-21), 4.15 (1H, dd, H-22), 4.02 (1H, dd, H-23), 1.55 (1H, m, H-24), 1.70 (1H, m, H-25), 0.82 (3H, d, H = 6.6 Hz, H-26), 0.84 (3H, d, H = 6.6 Hz, H-27), 1.26 & 0.83 (2H, m, H-28), 0.86 (3H, t, H = 6.5 Hz, H-29), 4.20 (1H, d, H-1), 2.87 (1H, dt, H-2), 3.17 (1H, dt, H-3), 3.02 (1H, m, H-4), 3.06 (1H, m, H-5), 4.45 & 4.30 (2H, m, H-6), 3.54 (1H, s, 2′OH), 3.54 (1H, s, 3′OH), 3.42 (1H, s, 4′OH), 3.60 (1H, s, 5′OH).¹³C NMR (100 MHz, CDCl₃) 37.7 (C-1), 31.7 (C-2), 71.8 (C-3), 42.4 (C-4), 140.8 (C-5), 121.7 (C-6), 31.9 (C-7), 31.9 (C-8), 50.2 (C-9), 36.2 (C-10), 21.1 (C-11), 39.8 (C-12), 42.3 (C-13), 56.7 (C-14), 26.1 (C-15), 28.3 (C-16), 56.1 (C-17), 19.0 (C-18), 11.9 (C-19), 35.40 (C-20), 19.4 (C-21), 33.29 (C-22), 26.0 (C-23), 45.9 (C-24), 29.3 (C-25), 20.8 (C-26), 18.9 (C-27), 22.7 (C-28), 12.0 (C-29), 102.2 (C-1′), 72.32 (C-2′), 75.56 (C-3′), 73.02 (C-4′), 76.63 (C-5′), 63.62 (C-6′). NMR data were comparable to those reported in the literature (40). The compound was identified as stigmasterol glucoside (Figure 1a).

**Compound 2:** Yellow amorphous solid; *C₁₅H₁₀O₁₆*; *Rf*, 0.45; mp 178–182°C; IR *ν*~max~ cm⁻¹: 3642.12 (OH), 2620.15 (CH), 1220.24 (C–O); ¹H NMR (500 MHz, DMSO-d₆): δ 7.23 (1H, dd, H, d, J = 8.0 Hz, H-6′), 7.12 (1H, d, J = 2.5, H-2′), 6.48 (1H, d, J = 8.0 Hz, H-5′), 6.65 (1H, d, J = 2.3 Hz, H-8), 6.53 (1H, s, H-3), 5.46 (1H, d, J = 2.2 Hz, H-6); ¹³C NMR (125 MHz, DMSO-d₆): δ 178.6 (C-4), 165.1 (C-7), 162.2 (C-2), 160.2 (C-5), 156.32 (C-9), 148.3 (C-4), 144.7 (C-3), 120.2 (C-1), 117.02 (C-6), 110.5 (C-5), 112.3 (C-2), 102.2 (C-1′), 72.32 (C-2′), 75.56 (C-3′), 73.02 (C-4′), 76.63 (C-5′), 63.62 (C-6′).
102.2 (C-10), 100.02 (C-3), 97.7 (C-6), 92.4 (C-8). NMR data were comparable to those reported in the literature (41). The compound was identified as luteolin (Figure 1b).

2.6. Preparation of Plectranthus asirensis biomass

Leaves and stems of *P. asirensis* were collected after harvest. Samples were thoroughly washed under water current and then oven dried at 90°C for 10 days. The resulting biomass were finely ground and sieved to acquire a homogenous powder, which then washed with 0.01 mol L⁻¹ hydrochloric acid (HCl) followed by distilled water. Ethanolic extract of *P. asirensis* biomass was then used for the synthesis of silver nanoparticles in the presence of micellar medium.

2.7. Synthesis of silver nanoparticles using Plectranthus asirensis biomass

Eco-friendly and micellar-aid green synthesis of AgNPs was conducted by reducing silver nitrate using an ethanolic extract of *P. asirensis* biomass and 1.0% of micellar substance sodium dodecyl sulfate (SDS). The synthesis of AgNPs was conducted by mixing 50 mL of 1.0 mmol L⁻¹ silver nitrate solution with 5.0 mL of 1.0% SDS and 3.0 mL of *P. asirensis* biomass with continuously stirring for 60 min in darkness and at 80°C. Yellowish to the brown color product was observed, which indicated the formation of Ag⁺ ion. The influence of reaction time was studied at different intervals (10, 15, 20, 25, 30, 35 and 40 min). The obtained solution was subjected to centrifugation at 3500 rpm for 5 min. Microscopic and spectroscopic techniques were used to characterize the formed AgNPs.

2.8. Spectroscopic and microscopic characterization of AgNPs

Samples were analyzed using Ultrospec 2100® pro spectrophotometer (Biochrom, England). JEM-1400 plus TEM and JSM-7610F SEM were used to characterize the size and morphology of the formed AgNPs at a 100 kV accelerating voltage and 20000 X magnification.

![Figure 1. Chemical structures of (a) compound 1: stigmasterol glucoside and (b) compound 2: luteolin.](image)

![Figure 2. Illustration of sequential injection analysis chemiluminescence system.](image)
2.9. General SIA-CL procedure

The SIA-CL measurements were carried out under a PC control to verify the optimum precision and control of valves and pump movement (Figure 2). All lines of the system were filled with the carrier solution (distilled water) to remove any air bubbles, then the studied solution and reagents were aspirated. The SIA-CL system (luminol–ferricyanide (III)-AgNPs) was used for the detection of two different natural extracted products (stigmasterol glucoside and luteolin). A mixture of the selected reagents for the suggested system was aspirated as 50 µL of luminol, 40 µL AgNPs and 60 µL of potassium ferricyanide (III) under a flow rate of 100 µL s$^{-1}$ and 30 µL of each sample.

2.10. The suggested control program

The SIA control program was exploited to execute all calibration measurements and experimental investigation of each of stigmasterol glucoside and luteolin. The standard program sequence was listed in Table 1. Each signal cycle was carried out for 45 s and the final sample throughput was 80 h$^{-1}$.

2.11. The plotted calibration graphs

Calibration graphs for the detection of stigmasterol glucoside and luteolin were plotted under optimum conditions using the CL intensity vs. the concentration of the tested stigmasteryl glucoside and luteolin solutions at 10 experimental points. Triplicate detection was performed for each aspirated sample. Least square linear regression was utilized for graphs fitting.

2.12. Antioxidant activity using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

Antioxidant activity was determined using DPPH, based on the protocol previously reported by (Brand-Williams et al. 1995) (42). Approximately 50 mL of different stigmasteryl glucosides and luteolin concentrations were mixed with 150 mL DPPH (a final concentration of 100 mmol L$^{-1}$). The reaction was incubated for 30 min at 37° C in dark, and the optical density was determined at 515 nm. Antioxidant activity was expressed as IC$_{50}$. Quercetin and α-tocopherol were used as standard antioxidants for comparison. A non sample DPPH solution was used as control. Quadruplicates were used to determine all curves.

3. Results and discussion

3.1. Spectroscopic characterization of AgNPs

UV-vis spectroscopic detection was carried out to evaluate the optical properties of the prepared AgNPs using an ethanolic extract of P. asirensis biomass. A significant absorption peak at 420 nm was detected (Figure 3).

Table 1. The controlled SIA-CL program for detection of the extracted stigmasterol glucoside and luteolin.

| Device               | Command              | Parameter | Action                                      |
|----------------------|----------------------|-----------|---------------------------------------------|
| Loop start ($) 1     | Counter clockwise    | % 50      | Pump filled with carrier                    |
| Next sample          | Syringe pump         | Delay (s) | 45                                          |
| Peristaltic pump     | Detector             | Peristaltic pump off |                                      |
| Peristaltic pump     | Syringe pump         | Valve position IN |                                      |
| Syringe pump         | Syringe pump         | Set flow rate (µL s$^{-1}$) | 100                                      |
| Syringe pump         | Syringe pump         | Aspirate (µL) | 1500                                      |
| Multiposition valve  | Multiposition valve  | Delay until done |                                      |
| Syringe pump         | Syringe pump         | Set valve position | 3                                       |
| Syringe pump         | Syringe pump         | Set flow rate (µL s$^{-1}$) | 100                                      |
| Multiposition valve  | Syringe pump         | Aspirate (µL) | 50                                        |
| Syringe pump         | Syringe pump         | Set valve position | 2                                       |
| Syringe pump         | Syringe pump         | Set flow rate (µL s$^{-1}$) | 100                                      |
| Multiposition valve  | Multiposition valve  | Delay until done |                                      |
| Syringe pump         | Syringe pump         | Set valve position | 4                                       |
| Syringe pump         | Syringe pump         | Set flow rate (µL s$^{-1}$) | 100                                      |
| Syringe pump         | Syringe pump         | Aspirate (µL) | 60                                        |
| Multiposition valve  | Multiposition valve  | Delay until done |                                      |
| Syringe pump         | Detector             | 7         | Potassium ferricyanide 5.0 × 10$^{-4}$ mol L$^{-1}$ |
| Syringe pump         | PMT                  | Set flow rate (µL s$^{-1}$) | 100                                      |
| Syringe pump         | Syringe pump         | Start scan |                                      |
| PMT                  | Syringe pump         | Empty     |                                            |
| PMT                  | Refresh plat         | Stop scans |                                            |
| Loop end             |                      |           |                                            |
According to the literature values (43) this distinct peak indicates the successful formation of AgNPs with a particle size of approximately 20 nm. The crystalline feature of the biosynthesized AgNPs was confirmed using XRD spectrum (Figure 4a). The recorded peak values were observed at $2\theta = 37.91^\circ$, $45.15^\circ$, $68.59^\circ$, and $79.57^\circ$ relative to the Ag (111), Ag (200), Ag (220) and Ag (311) lattice planes of the face centered cubic crystal structure of AgNPs.

The purity of the synthesized AgNPs was ensured using Fourier-transform infrared (FT-IR), spectroscopy. The recorded FT-IR spectrum showed two main bands detected at 530 and 1360 cm$^{-1}$ due to the presence of Ag$^+$ and N–O from AgNO$_3$. However, other bands at 3310 and 2910 cm$^{-1}$ were attributed to the N–H stretching and O–H stretching bands, respectively, due to the adsorption of water on the surface of AgNPs. Other two vibrational bands were recorded at 1635 and 1018 cm$^{-1}$, which may be attributed to the carbonyl vibration band and typical C-4-OH of a glucose residue of disaccharides, respectively. The results of the FT-IR spectroscopic study confirmed the reduction of silver nitrate into AgNPs (Figure 4b).

### 3.2. Microscopic characterization of AgNPs

The surface morphology and distribution of the synthesized AgNPs were studied using TEM and SEM. It was observed that the AgNPs is fairly distributed and their particle size was, approximately, 20 nm (Figure 5a). Moreover, the SEM image was used to investigate the surface morphology of the formed nanoparticles (Figure 5b).

### 3.3. Optimization of CL conditions

Three different types of CL reagents such as luminol, lucigenin and sodium sulfite were tested, to select a suitable CL reagent for the proposed CL system. It was found that...
luminol provided the highest CL signal (Figure 6a). Also, the suitable alkaline medium was investigated using $1.0 \times 10^{-4} - 1.0 \times 10^{-1}$ mol L$^{-1}$ of sodium hydroxide, potassium hydroxide, sodium carbonate, ammonium hydroxide, and sodium bicarbonate. The strongest signal was observed using luminol in $1.0 \times 10^{-2}$ mol L$^{-1}$ of sodium hydroxide (Figure 6b). Furthermore, the oxidizing agent employed in the CL system was selected by comparing the CL signals between sodium periodate, potassium permanganate, hydrogen peroxide, potassium ferricyanide (III), potassium dichromate and Ce (IV) ammonium sulfate. Potassium ferricyanide (III) was selected as an excellent oxidizing agent in the presence of AgNPs (Figure 6c).

To adjust the CL system, different concentrations in the range of $1.0 \times 10^{-6} - 1.0 \times 10^{-2}$ mol L$^{-1}$ of each of luminol and potassium ferricyanide (III) were studied. Maximum CL signals were obtained using $5.0 \times 10^{-5}$ mol L$^{-1}$ of luminol and $5.0 \times 10^{-4}$ mol L$^{-1}$ of potassium ferricyanide (III) for the detection of stigmasterol glucoside and luteolin in the presence of AgNPs (Figures 6d).

### 3.4. Effect of flow and aspiration rates

To accomplish the SIA-CL system for stigmasterol glucoside and luteolin detection, some physical parameters including the volume of AgNPs, flow rate and aspirate volumes of the sample and reagents were adjusted. Automatic control of the SIA system using FIAlab software version 5.9.321 was used for the aspirated volume of both samples and reagents. The outcome results of SIA-CL determination of the studied materials were 50 μL of luminol, 60 μL of potassium ferricyanide (III) and 30 μL of each stigmasterol glucoside and luteolin in the presence of 1.0 mL of AgNPs at a 100 μL s$^{-1}$ flow rate.

### 3.5. Possible CL reaction mechanisms

The CL reaction involves a highly oxidizing species such as potassium ferricyanide (III). In the luminol-potassium ferricyanide system and, under the optimum CL reaction conditions, emitted light intensity was increased with short duration time in the presence of AgNPs indicating the catalytic activity of the added nanoparticles. As shown in Scheme 1, the reduction of luminol radicals was accomplished on the surface of the nanoparticles during the exchange interaction between the unpaired electrons of luminol and the conduction band electrons on the nanoparticles.

Under optimal conditions, AgNPs of 20 nm size displayed the highest CL intensity lasting for long duration of time. This effect can be attributed to the increase in surface area and surface electron density in the catalytic reaction involving NPs. It has also been reported that organic compounds containing hydroxyl (OH) and amino (NH$_2$) groups can greatly interact with nanoparticles leading to the enhancement or inhibition of CL signal amplification (44). Therefore, the present study focused on the possibility of stigmasterol glucoside and luteolin to react with the prepared nanoparticles and detected using SIA-CL system.

### 3.6. Analytical detection of stigmasterol glucoside and luteolin in real extract

The SIA-CL system was used to detect the isolated compounds in their standard form and real samples. It was
found that the suggested method displayed linear concentration ranges of 5–100 and 2–120 µg mL$^{-1}$ with $r = 0.9995$ and 0.9996 for stigmasterol glucoside and luteolin, respectively (Figures 7a and b).

The developed SIA-CL technique was also employed for the determination of both compounds in real extract from the aerial parts of the *P. asiensis* plant. The obtained results were 10.05 µg mL$^{-1}$/5 mg mL$^{-1}$ and 85.8 µg mL$^{-1}$/5 mg mL$^{-1}$ with total yields of 1.7% and 0.2% for stigmasterol glucoside and luteolin, respectively.

### 3.7. Antioxidant activity

The determined antioxidant activity estimated as IC50 values of DPPH radical scavenging activity showed high free radical scavenging activity for both luteolin and stigmasterol. Even though both compounds exhibited potent activity, the IC50 values of luteolin (12.725 ± 0.326) were slightly lower than those of the stigmasterol (14.541 ± 0.225) under the conditions of this study. It has been reported that reactive oxygen species (ROS) contribute to the onset of the inflammatory response by activating the nuclear factor-kappa B transcription factor that mediates the inflammation gene expression (45). Since luteolin and stigmasterol were both known to exhibit an anti-inflammatory activity (46,47), the high antioxidant activity, could explain, in part, their anti-inflammatory effects, as oxidative stress is closely associated with inflammation.
4. Conclusion

The present study focused on the isolation of bioactive constituents, stigmasterol glucoside and luteolin from an ethanolic extract of aerial parts of *P. asirensis*. Quantitative estimation of the isolated constituents was performed in the ethanolic extract of *P. asirensis* by environmentally friendly, cost effective and highly sensitive Ultrasensitive SIA-CL system using AgNPs. The suggested SIA-CL provided excellent sensitivity and reliability to quantify the isolated compounds in their real samples. The antioxidant activity of the isolated stigmasterol glucoside and luteolin was studied and they found to be exhibiting high antioxidant activity explaining their high anti-inflammatory activity.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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