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Full Length Research Paper

Utility of silver nanoparticles for the analysis of diosmin and rutin in *Persicaria salicifolia* extract, authentic and pharmaceutical dosage forms monitored with their haemostatic activity

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The present work was conducted to evaluate a simple effective technique for spectrophotometric determination of two flavonoids namely diosmin (1) and rutin (2) in the isolated fractions obtained from *Persicaria salicifolia*, authentic and pharmaceutical dosage forms, the method is based on the reduction of Ag^+ cations to silver nanoparticles (Ag-NPs) by the effect of these flavonoids in the presence of polyvinylpyrrolidone (PVP) as a stabilizing agent which result in production of intense brown colour corresponding to the formation of the surface plasmon resonance of produced silver nanoparticles. The plasmon absorbance of the Ag-NPs allows quantitative spectrophotometric determination of these flavonoids where calibration curves derived from the changes in absorbance at λ=415 and 432 nm were linear with concentration range of diosmin and rutin 1-8 and 0.5-7.0 µg/mL while their detection limits (3σ) were 0.26 and 0.12, respectively. The method was applied successfully for the determination of flavonoids in extract, authentic and pharmaceutical dosage forms. The haemostatic activity of the total methanol extract, isolated fractions, authentic and dosage forms were investigated for their usual and silver nanoparticulated forms using tail bleeding time and amount of bleeding assays in rats, where the nanoparticulated samples exhibited more significant activities.

Key words: *Persicaria,* diosmin, rutin, silver nanoparticles, spectrophotometry, haemostatic.

INTRODUCTION

Polygonaceae is a family of flowering plants comprising about 1200 species containing many important medicinal species (Mabberley, 2008; Heywoodet al., 2007); it consists of about 48 genera, among them the genus *Persicaria* include 100 species distributed worldwide (Tantawy et al., 2005). In Egypt, Polygonaceae includes about 22 wild species under six genera among them are seven species belonging to genus *Persicaria* (Boulos,

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2009). *Persicaria salicifolia* (BROUSS. ex WILLD.) ASSENOV is a hydrophyte plant growing along the Nile Delta (Shaltout et al., 2010). Phytochemical investigation of *P. salicifolia* revealed the existence of various flavonoids (Hussien and Mohamed, 2013; EL-Magly., 2017) while biological investigations revealed their antioxidant and cytotoxic activities (Hussien and Mohamed, 2013).

Flavonoids have historically been used in Chinese and Ayurvedic medicines for their versatile biological effects; they affects skin integrity, brain function, blood sugar and blood pressure, and moreover, they exhibit antioxidant and anti-inflammatory activities (Proestos et al., 2006), and among these flavonoids are the flavone glycoside diosmin (3', 5', 7-trihydroxy-4'-methoxyflavone 7-rutinoside) (Figure 1a) and rutin (5, 7, 3', 4'tetrahydroxy flavonol-3-rhamnoglucoside) (Figure b) (Alam et al., 2016).

Several techniques have been reported for determination of diosmin in plant extracts, biological fluids and dosage forms, the majority of which are chromatographic in nature (Kanaze et al., 2003; Janeczko et al., 2003), in addition to capillary electrophoretic (Sawalha et al.,2009; Aturki and Sinibaldi, 2003), electrochemical (El-Shahawi et al., 2006), spectrophotometric (Moldovan et al., 2010; Mehra,1990) and spectrofluorimetric (Mohamed and Tawakkol, 2013) techniques meanwhile various analytical techniques were applied for the determination of rutin in raw material, pharmaceuticals and biological fluids and they include HPLC (Kuntic et al., 2007; Mauludin and Müller, 2009), voltammetry (Ensafi and Hajian, 2006; Volikakis and Efstathi, 2009) and spectrophotometry (Januja et al., 2009; Filtik et al., 2002).

Nanoparticles made of noble metals have been the focus of research for many decades as a result of their intriguing optical properties; among the noble metal nanoparticles, silver nanoparticle gained special interest because of their advantageous properties concerning stability and conductivity (Popescu et al., 2010; Baruwati et al., 2009), in addition, they have been the subject of intensive research because of their unique and tunable surface plasmon resonance (SPR) properties when dispersed in liquid media exhibiting a strong characteristic UV-Vis extinction band that is not present in that of the bulk metal. This extinction band results when the incident photon frequency is resonant with the collective excitation of the conduction electrons and is known as the surface plasmon resonance (SPR). SPR excitation results in wavelength-selective absorption with extremely large molar extinction coefficients where the peak wavelength and intensity of the SPR spectrum is dependent upon the size, shape and inter-particle spacing of the nanoparticle as well as its own dielectric properties and those of its local environment including the substrate, solvent and adsorbates (Baruwati et al., 2009; Nezhad et al., 2010). Silver nanoparticles exhibit significant antibacterial, antiviral, antifungal, anti-inflammatory effects, in addition; they are used in cancer diagnosis and treatment (Ahmed et al., 2003).

Many reports have been published concerning bio-green synthesis of silver nanoparticles using plant extracts and they revealed that the nanoparticulated extracts were economic, energy efficient and cost effective (Logeswari et al., 2012; El-Hela et al., 2017), moreover they were utilized in several colourimetric determinations of many pharmaceuticals as nebivolol (Rahman et al., 2013), fexofenadine (Rahnama, 2013), catecholamines (Tashkhourian et al., 2011), etilefrine hydrochloride, fenoterol hydrobromide, salbutamol sulphate and estradiol valerate (Magda et al., 2015), fluroro-quinolones (Sayed et al., 2017), 5-fluorouracil (Safwata et al., 2016), antioxidants [Abdelhady and Badr., 2016) and antimicrobials offering sensitive and selective procedures.

The present study was conducted targeting bio-green synthesis of silver nanoparticles of the crude extract of *P. salicifolia* seeds (Figure 2), fractionated diosmin and rutin, their authentic and dosage forms; their characterization, pharmaceutical analysis utilizing very simple and sensitive colourimetric technique relying on their properties as active reducing agents participating in generation of silver nanoparticles with monitoring of their haemostatic activity.

MATERIALS AND METHODS

Plant material

P. salicifolia plants were collected in November 2015 from the Nile river irrigation canals, pond edges and irrigation canal bank of Ekhnaway, Tanta, El-Gharbia Governorate, a voucher specimen (C.S. # 0909) was deposited in a herbarium in Pharmacognosy Department, Faculty of Pharmacy, Al Azhar University, Cairo, Egypt. The plant seeds were separated, air-dried, powdered (2 mm mesh) and kept in tightly closed amber coloured glass containers protected from light at low temperature.

Chromatographic analysis

CC analysis was performed using Silica gel (C_{18} -reversed phase silica gel, 40-63 µm, 230-400 mesh, 90 A° pore size, Sigma-Aldrich), methanol, *n*-hexane, methylene chloride, ethyl acetate, *n*-butanol and acetic acid (Al-Gamhoria Co. for Chemicals, Egypt); PC analysis was performed on Whatmann No.1 sheets (Whatmann Ltd., Maidstone, Kent, and England); spot detection was carried out with the spraying with ferric chloride and aluminum chloride reagents; standard flavonoids; rutin and diosmin were supplied by Department of Pharmacognosy, Medical University of Gdansk, Poland.

Pharmaceutical analysis

Chemicals

The highest purity diosmin (99.2%) was obtained from Amriya for Pharmaceutical Industries, Alexandria, Egypt); Rutin, (99.7%) was

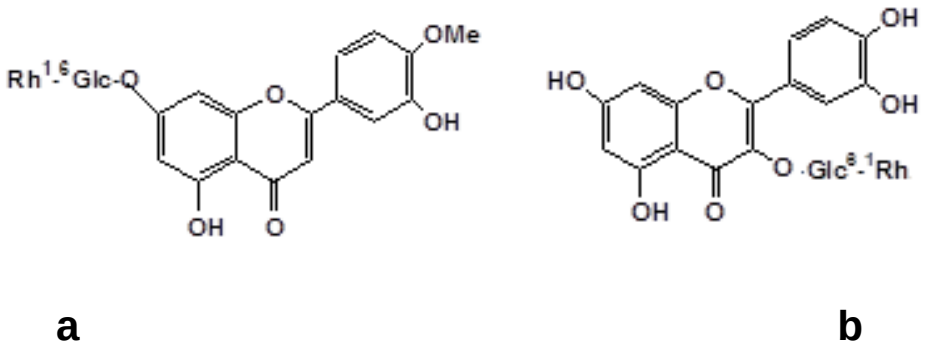

Figure 1. Chemical structures of diosmin (a) and rutin (b).

Figure 2. Seeds of *P. salicifolia.*

obtained from Kahira Pharmaceuticals and Chemical Industries Co., Cairo, Egypt); Silver nitrate, (0.02 M aqueous solution); polyvinylpyrrolidone (PVP), 0.14% aqueous solution and sodium hydroxide (0.0025 M aqueous solution) were also used.

Pharmaceutical preparations

Dioven® tablets containing 500 mg diosmin per tablet with Batch No.1147016 (B) was obtained from Amriya for Pharmaceutical Industries, Alexandria, Egypt and Ruta C 60® tablets containing 60 mg rutin per tablet with Batch No. 31304 was obtained from Kahira Pharmaceuticals and Chemical Industries Co., Cairo, Egypt).

Standard solutions

Solutions of 100 µg/mL of diosmin and rutin were prepared by dissolving 10 mg of the pure drug in bi-distilled water.

Biological studies

Animals

Adult albino rats (150-200 g) of either sex were purchased from The Animal House Laboratory, National Research Center, Cairo, Egypt, according to the protocol of the Institutional Animal Care and Use Committee (IACUC) and they were housed in an environmentally control room, maintained at uniform light and temperature conditions of and provided with food and water *ad libitum.*

Test material

Extract and fractions; total methanol extract, fractionated diosmin, fractionated rutin; authentic material; diosmin, rutin and their pharmaceutical dosage forms, all were tested in their ordinary and nanoparticulated forms.

Instrumentation

Soxhlet, vacuum oven (Vacucell, Einrichtungen GmbH), Genesys Spectrophotometer (Milton Roy, INC., Rochester, NY) for UV-Vis. Investigation of nanoparticles, field emission scanning electron microscope (SEM, JSM 6490A, Jeol, Tokyo, Japan), chromatographic glass jars, rotatory evaporator (BUCHI Rotavapor® R-210/R-215, Germany), Shimadzu - UV 1800 double beam UV-Visible spectrophotometer (Japan) with matched 1 cm quartz cells at 200-800 nm range was used for all absorbance measurements. Spectra were automatically obtained by Shimadzu UV-Probe 2.32 system software.

Extraction and fractionation

300 g of powdered *P. salicifolia* seeds were exhaustively extracted using soxhlet with 1000 mL of 80% (v/v) aqueous methanol for 1 h to yield the methanol extract which was subsequently filtered under vacuum through Whatmann No. 1 filter paper. The residue was re-extracted following the same procedure two more times; the filtered extracts were pooled together and concentrated under vacuum at 40°C to dryness to yield the collective methanol extract (52 g). 45 g of the methanol extract were suspended in sufficient volume of distilled water and successively fractionated using *n*-hexane, methylene chloride and ethyl acetate where each fraction was concentrated under reduced pressure at 40°C; they yielded 7.5 and 11 g, respectively. 8 g of the ethyl acetate fraction were chromatographed on 200 g silica gel packed on glass column (3 cm diameter, 0.75 m length), elution with gradient mode was carried out starting water 100% to methanol 100% at flow rate 10 mL min^{-1} to give 32 fractions; 100 mL each. The collected fractions were monitored with paper chromatography using S_1; *n*-butanol:acetic acid:water (4:1:5 v/v), upper layer, S_2; acetic acid:water (15:85 v/v) as solvent systems; the resultant spots were visualized with ferric

chloride (Markham, 1982) and aluminum chloride (Stahl, 1969) spray reagents for phenolic compounds and flavonoids, respectively, where similar fractions were pooled together.

Formation of silver nanoparticles

P. salicifolia methanol extract, fractionated diosmin and rutin, authentic diosmin and rutin as well as their pharmaceutical dosage forms were used to produce their silver nanoparticles as follows: in 5 mL volumetric flask appropriate equal volumes of $AgNO_3$ solution (0.1%) and 1% aqueous solution of each of the studied drugs were mixed, then PVP and appropriate amounts of NaOH were added, mixed and completed to 5 mL with bi-distilled water, heated in the water bath at 90^{o}C for appropriate times to allow the reduction processes to occur which were noticed by the colour change to brownish indicating the formation of the nanoparticles. The brown colour of the produced AgNPs is due to excitation of their surface plasmon resonance; this was observed first by naked eye and subsequently subjected to spectroscopic characterization. The obtained nanoparticles were purified through centrifugation at 10,000 rpm for 5 min, washed and dried in vacuum chamber for 24 h at 35°C, absorbance was measured at the suitable wavelength against reagent blank treated similarly (Praba et al., 2015).

Characterization of silver nanoparticles

The shape, size and morphology of the silver nanoparticles were determined using Scanning Electronic Microscopy (SEM) and Transmission Electron Microscopy (TEM).

Assay of tablets

For either Dioven® tablets or Ruta C 60® tablets, ten tablets were weighed, coat removed and pulverized into fine powder, specific quantity of powdered drugs equivalent to 10 mg pure drug were dissolved in distilled water, solutions were filtered and diluted to 100 mL with distilled water then further diluted to 10 µg/mL. Procedures were completed as in general procedures.

Experimental design for biological studies

One hundred and eighty rats of both sexes were divided into 15 groups; 12 rats each received various treatments [distilled water and tested drugs (10 mg kg $^{-1}$ b.wt. oral dose)] as follows; group 1: normal control administered distilled water (normal control); group 2: administered authentic diosmin; group 3: administered nanoparticulated authentic diosmin; group 4: administered authentic rutin; group 5: administered nanoparticulated authentic rutin; group 6: administered total extract; group 7: administered nanoparticulated total extract; group 8: administered fractionated diosmin; group 9: administered nanoparticulated fractionated diosmin; group 10: administered fractionated rutin; group 11: administered nanoparticulated fractionated rutin; group 12: administered diosmin dosage form; group 13: administered nanoparticulated diosmin dosage form, group 14: administered rutin dosage form; group 15: administered nanoparticulated rutin dosage form where six rats from each group were used for determination of bleeding time, the other six rats from each group were used for determination of the amount of bleeding, evaluations were carried out 24 h after administration of the last dose.

Bleeding time

Bleeding times were determined as follows; each rat was placed in a standard plastic restraining cage with the tail hanging out freely, bleeding time was assessed by cutting the tip of the tail with a sharp pair of surgical scissors (2-3 mm), the tail was placed in an isotonic saline solution with pH 7.4 maintained at 37 ºC immediately after the cut was inflicted. Bleeding time was calculated through a stopwatch was started simultaneously with the immersion of the tail in the saline solution where the time taken was the time from appearance of the first drop of blood to the time when the bleeding stopped completely (Rajasekaran et al., 2010).

Amount of bleeding

The quantity of bleeding was measured as follows; rats were placed individually in a standard plastic restraining cage with the tail allowed to hang out freely, the tip of the tail of each rat was cut (4-5 mm) with a sharp pair of scissors and a stopwatch started immediately, a pre-weighed blotting paper was used to collect all drops of blood that flowed from the site of the inflicted injury, the blotting paper was re-weighed after the appearance of the last drop of blood, the difference in weight of the dry and wet blotting paper was taken as the amount of bleeding (Cipil et al., 2009; Yalcinkaya et al., 2011).

Statistical analysis

Results were expressed as means ± SEM, significance was determined using students t-test and paired mode where results were regarded as significant at P≤0.001 and P≤0.0001(Elliott and Woodward, 2007).

RESULTS AND DISCUSSION

Fractionation and purification of ethyl acetate fraction of *P. salicifolia* seeds was carried out resulting in isolation of two major flavonoids and the data gained was as follows:

Compound 1 (35 mg): Yellow amorphous powder [MeOH]; UV λ_{max} (MeOH) nm: 260, 366, λ_{max} (MeONa) nm: 273, 315, 412, λ_{max} ($AlCl_3$) nm: 276, 305, 439, λ_{max} ($AlCl_3$/HCl) nm: 275, 303, 409, λ_{max} (AcONa) nm: 277, 320, 395, λ_{max} (AcONa/boric acid) nm: 261, 389; ^{1}H NMR (DMSO-d_6, 500 MHz) δ12.5 (OH), 7.54 (1H, br s, H-2`), 7.52(1H, d, J=8.0 Hz, H-6`), 6.83 (1H, d, J=8.0 Hz, H-5`), 6.45 (1H, br s, H-8), 6.22 (1H, br s, H-6), 5.40 (1H, d, J=7.6 Hz, H-1``), 4.39 (1H, d, J=2.5 Hz, H-1```), 3.05-3.38 (10H, m, H-2``-H-6`` of glc and H-2```-H-5``` of rha), 0.95 (1H, d, J=6.0 Hz, H-6``); $^{13}$C NMR (DMSO-$d_6$, 125 MHz) δ177.74 (C, C-4), 164.72 (C, C-7), 161.49 (C, C-5), 157.17 (C, C-2),156.75 (C, C-9),148.87 (C, C-4`), 145.18 (C, C-3`), 133.68 (C, C-3), 122 (CH, C-6`), 121.58 (C, C-1`), 116.60(CH, C-5`), 115.76 (CH, C-2`), 105 (C, C-10), 101.57 (CH, C-1``), 101.16 (CH, C-1```), 99. 2 (CH, C-6), 94.14 (CH, C-8), 76.83 (CH, C-3``), 76.19 (CH, C-5``), 74.43 (CH, C-2``), 72.23 (CH, C-4```), 70.92 (CH, C-3```), 70.69 (CH, C-2```), 70.36 (CH, C-4``), 68.61 (CH, C-5```), 67.40 (CH2, C-6``), 18.11 (CH3, C-6```); HRESI-MS (+): m/z 629.1560 $[M^{+}Na]^{+}$ (calcd. 631.1639 for $C_{27}H_{30}O_{16}$ Na) leading to the formula $C_{27}H_{30}O_{16}$; this compound was identified as quercetin-3-O-α-L-rhamnosyl-β-glucopyranoside (Rutin) according to previously

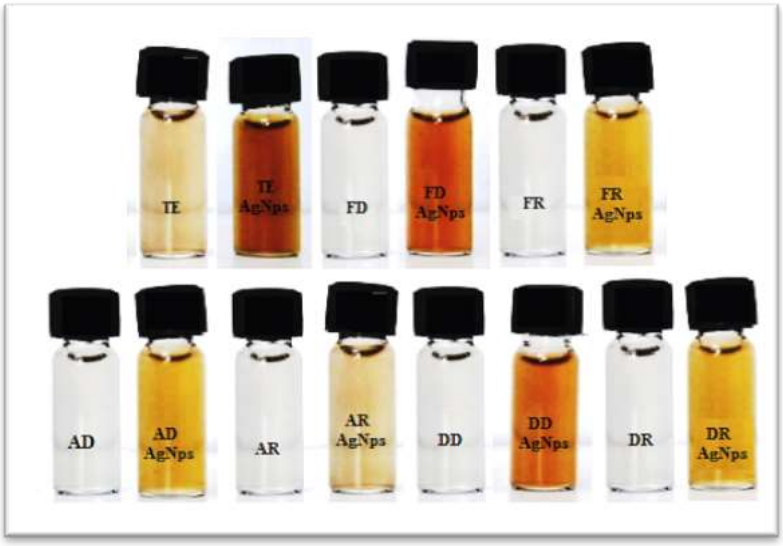

Figure 3. Colour changes when cited drugs were mixed with $AgNO_3$ solution: authentic diosmin (AD), authentic rutin (AR), total extract (TE), fractionated diosmin (FD), fractionated rutin (FR), dosage form rutin (DR), dosage form diosmin (DD) and their corresponding AgNps.

published spectral data and co-chromatography with authentic sample (Mabry et al., 1970; Agrawal, 1989).

Compound 2 (38 mg): White amorphous powder [MeOH]; UV λ_{max} (MeOH) nm: 260, 366, λ_{max} (MeONa) nm: 273, 315, 412, λ_{max} ($AlCl_3$) nm: 276, 305, 439, λ_{max} ($AlCl_3$/HCl) nm: 275, 303, 409, λ_{max} (AcONa) nm: 277, 320, 395, λ_{max} (AcONa/boric acid) nm: 261, 389; ^{1}H NMR (500 MHz, DMSO-d_6) δ (ppm) = 12.93 (1H, s, OH-5), 9.45 (1H, s, OH-3´), 7.58 (1H, dd, J = 8.6, 2.3 Hz, H-6´), 7.45 (1H, d, J = 2.3 Hz, H-2´), 7.14 (1H, d, J = 8.6 Hz, H-5´), 6.83 (1H, s, H-3), 6.77 (1H, d, J = 2.1 Hz, H-8). 6.47 (1H, d, J = 2.1 Hz, H-6) , 5.08 (1H, d, J = 7.4 Hz, H-1´´), 4.55 (1H, d, J = 1.1 Hz, H-1´´´), 3.1-3.8 (sugar protons) 3.87 (3H, s, OMe-4'), 1.08 (3H, d, J = 6.2 Hz, H-6´) ; ^{13}C NMR (125 MHz, DMSO-d_6) δ (ppm) = 181.92 (C-4), 164.20 (C-2), 162.94 (C-7), 161.17 (C-5), 156.94 (C-9), 151.33 (C- 4´),146.79 (C-3´) , 122.89 (C-1´), 118.92 (C-6´),113.12 (C-2´), 112.29 (C-5´), 105.9 (C-10), 103.8 (C-3), 99.94 (C-6), 94.81 (C-8), 100.49 (C-1´´´), 99.6 (C-1´´), 76.25 (C-3´´), 75.6 (C-5´´), 73.09 (C-2´´), 72.04 (C-4´´´), 70.73 (C-4´´), 70.3 (C-2´´´), 69.59 (C-3´´´), 68.29 (C-5´´´), 66.03 (C-6´´), 17.74 (C-6´´). HRESI-MS (+): m/z 631.1663 $[M^+Na]^+$ (calcd. 631.1639 for $C_{28}H_{32}O_{15}Na$) which led to the formula $C_{28}H_{32}O_{15}$ for this molecule. This compound was identified as 3', 5, 7-trihydroxy-4'-methoxyflavone-7-rutinoside (diosmin) according to previously published spectral data and co-chromatography with authentic sample (Mabry et al., 1970; Agrawal., 1989).

Concerning the preparation of silver nanoparticles in this study, an aqueous $AgNO_3$ solution including polyvinylpyrrolidone (PVP), as stabilizer, in an alkaline medium was used, then diosmin and rutin were added acting as effective reducing agents for the reduction of silver metal salt (Ag^+) to the Ag-NPs manifested by the brown colour produced upon completion of the reaction (Figure 3). The absorbance of reducing agents exhibited no absorbing peak in visible region (380-700 nm) meanwhile, upon addition of the cited drugs which act as reducing agent, silver ions were reduced to silver nanoparticles and then the absorbance characteristic to the plasmon of the Ag-NPs was observed (415 and 432 nm) for diosmin and rutin, respectively (Table 1, Figures 4 and 5).

Effect of NaOH concentration

The influence of pH on Ag^+ reduction by the cited drugs is expected since they have a hydroxyphenyl group which can lose H^+ during oxidation and *O*-quinone formation process. Because buffered condition failed to obtain silver nanoparticles, NaOH was added to provide enough alkalinity. By addition of NaOH, absorbance increases up to a known concentration of NaOH then decreases the formation of black precipitate which might be due to the Ag_2O formation. Thus, 1 mL of 0.0025 M NaOH was selected for each diosmin and rutin (Figure 6).

Effect of Silver nitrate concentration

Maximum absorbance values were obtained using 0.7 and 1.0 mL of 0.02 M silver nitrate for diosmin and rutin, respectively (Figure 7).

Table 1. Analytical parameters for determination of diosmin and rutin through silver nanoparticles formation.

Parameter	Diosmin	Rutin
$\lambda_{max.}$(nm)	415	432
Volume of Silver nitrate (0.02 M), mL	0.7	1.0
Volume of PVP (0.14%), mL	0.7	0.5
Volume of NaOH (0.0025 M), mL	1.0	1.0
Temperature, ^{o}C	90	90
Time of reaction, min.	40	30
Beer's law limits (µg/mL)	1.0-8.0	0.5-7.0

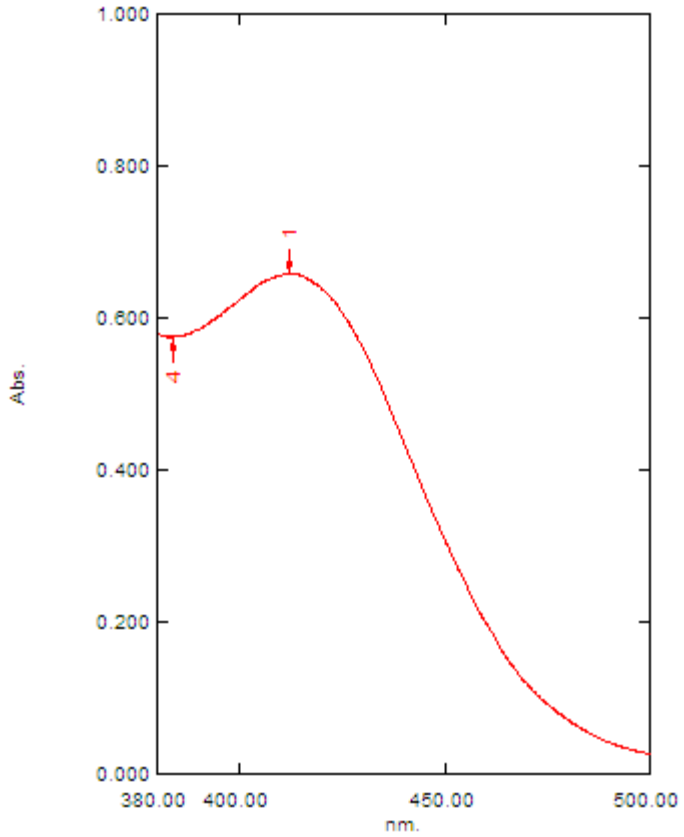

Figure 4. Absorbance spectra of the silver nanoparticles formed in the presence of 5 µg/mL Diosmin.

Effect of Stabilizer type and concentration

An important issue in the preparation of metal nanoparticles is the choice of the capping agent used to protect or stabilize the nanoparticle colloidal metals from agglomeration. Size and morphologies of nanoparticles depends significantly on capping materials. Nanoparticles stabilization is achieved according to the two basic modes: electrostatic and steric stabilization (Januja et al., 2009). Electrostatic stabilization is caused by the columbic repulsion between particles, caused by the electrical double layer formed by ions adsorbed at the particle surface (for example sodium citrate) and the corresponding counter ions. Steric stabilization is achieved because of the coordination of steric ally demanding organic molecules and polymers that act as protective shields on the metallic surface (for example PVP). In this study, PVP and sodium citrate were selected as the stabilizer for preventing of silver nanoparticles agglomeration in which the PVP was better

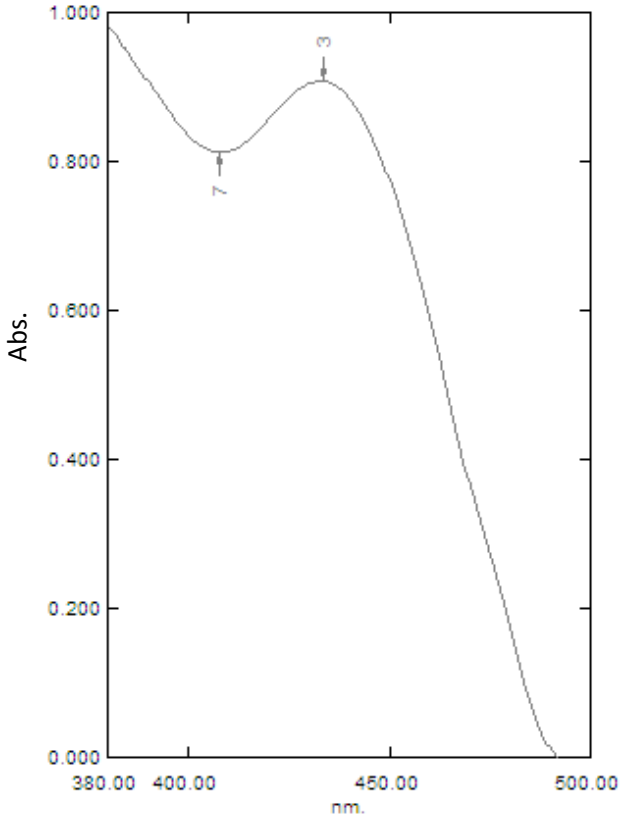

Figure 5. Absorbance spectra of the silver nanoparticles formed in the presence of 6 µg/mL Rutin.

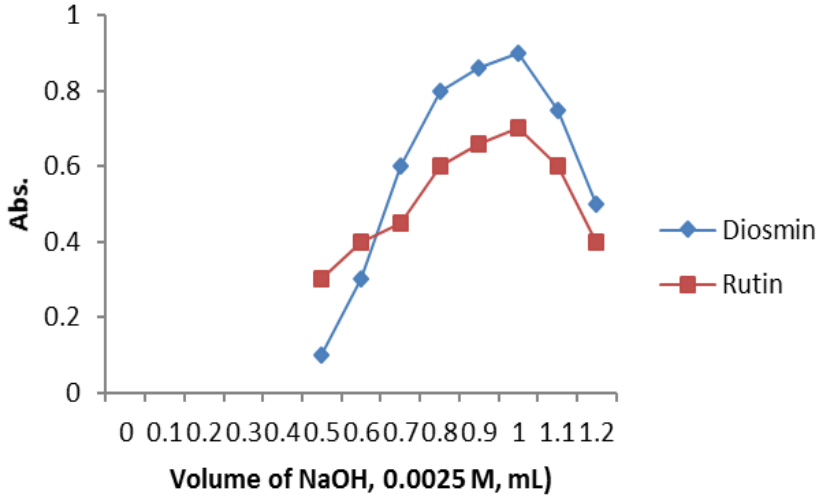

Figure 6. Effect of NaOH on the absorbance spectra of the reaction product between AgNPs and the studied drugs in the presence of PVP.

used compared to sodium citrate. 0.7 and 0.5 mL of 0.14 % PVP were optimum for diosmin and rutin, respectively (Figure 8).

Effect of temperature and time of heating

Heating in water bath at 90°C for 40 and 30 min were

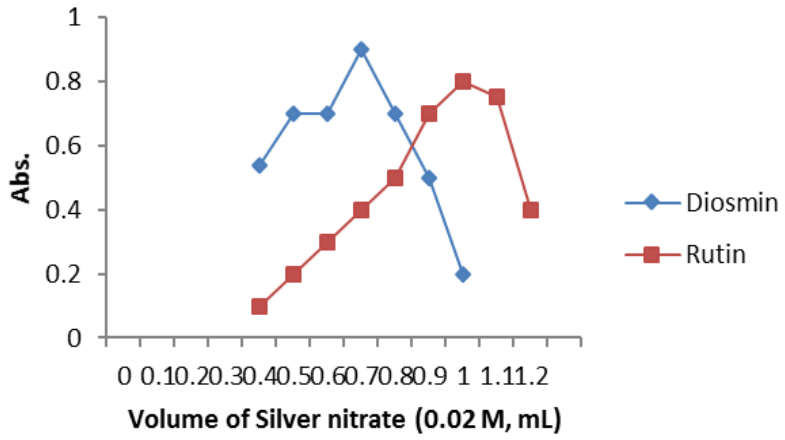

Figure 7. Effect of AgNPs concentration on the absorbance spectra of the reaction product between AgNPs and the studied drugs in the presence of PVP.

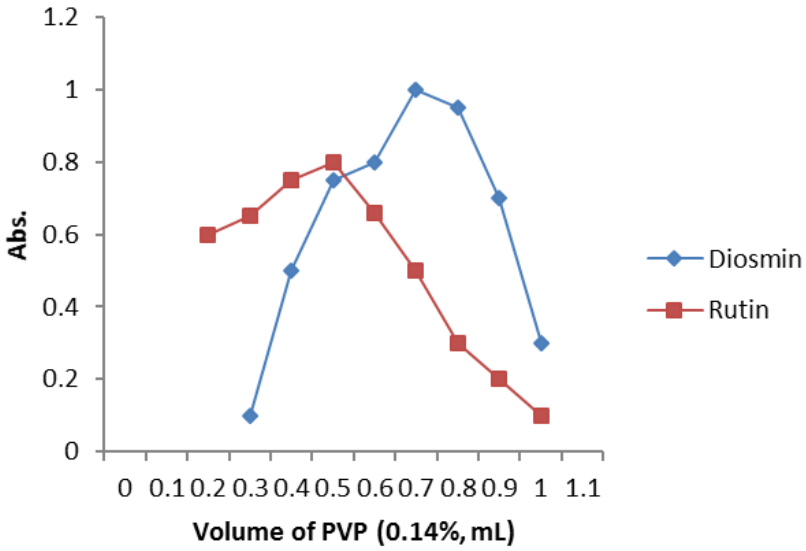

Figure 8. Effect of PVP concentration on the absorption spectra of AgNPs with studied drugs.

sufficient to produce maximum color intensities for diosmin and rutin, respectively (Figure 9).

Method validation

Linearity

Under the described experimental conditions, standard calibration curves with good linearity for silver nanoparticles formed using authentic diosmin and rutin were constructed by plotting absorbance against concentration (Figures 10 and 11).

A linear correlation was found, the concentration ranges, correlation coefficient, intercept and slope for the calibration curve were calculated, also relative standard deviation, detection and quantification limits were calculated for authentic and fractionated diosmin and rutin (Tables 2 to 4). The validity of the proposed method was assessed by its application to the determination of the studied drugs in their pharmaceutical preparations (Tables 5 and 6).

Student's t-test and F-test (at 95% confidence level) were applied to the results obtained compared with that obtained from reported methods (Moldovan et al., 2010; Younes et al., 2014); the results showed that there were

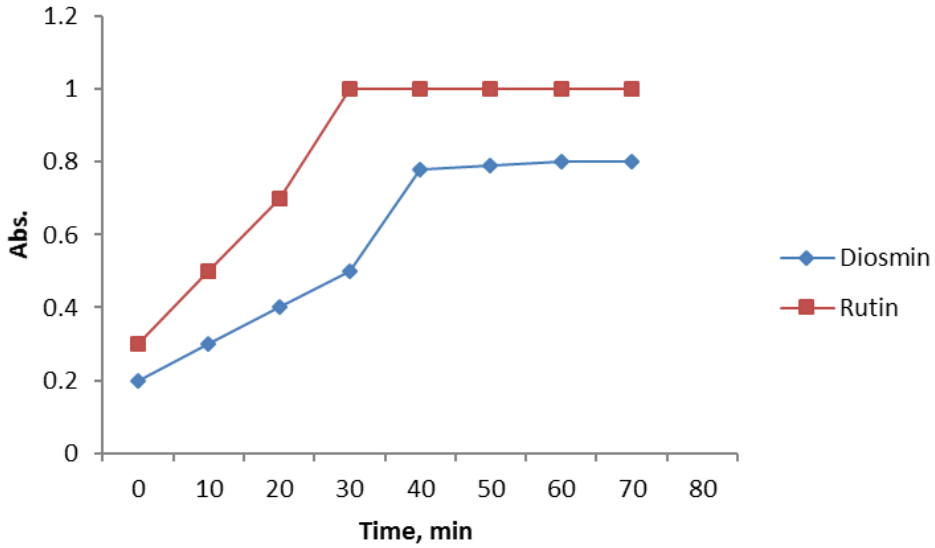

Figure 9. Effect of reaction time on the absorption spectra of the corresponding reaction of the studied drugs.

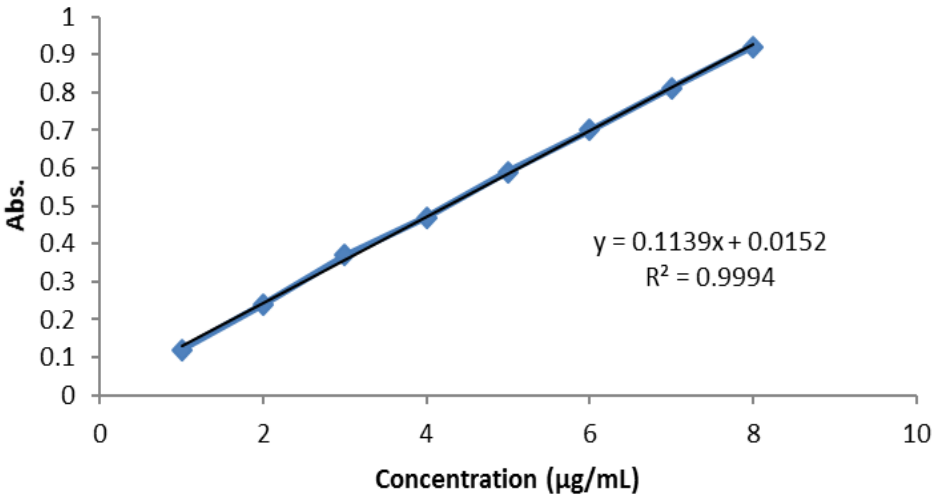

Figure 10. Calibration curve of diosmin reacted with Ag-NPs.

no significant differences between the proposed and reported methods. Results of different statistical treatment of the data (Table 7).

Accuracy and precision

Accuracy and precision were carried out by six determinations at two different concentrations of the two drugs in the same day (intra-day), and in six different days (inter-day). Percentage relative standard deviation (R.S.D. %) was used as precision. The results of accuracy and precision show that the proposed methods have good repeatability and reproducibility (Table 8).

Nanoparticle characterization

SEM analysis was employed to determine the surface morphology and the topography of synthesized silver nanoparticles where the size of silver nanoparticles ranged from 28 to 33 nm, with an average size 30 nm. The gained SEM images showed that the gained silver nanoparticles were mostly spherical in shape while TEM analysis revealed that most particles were obviously spherical in shape and well dispersed, with an average

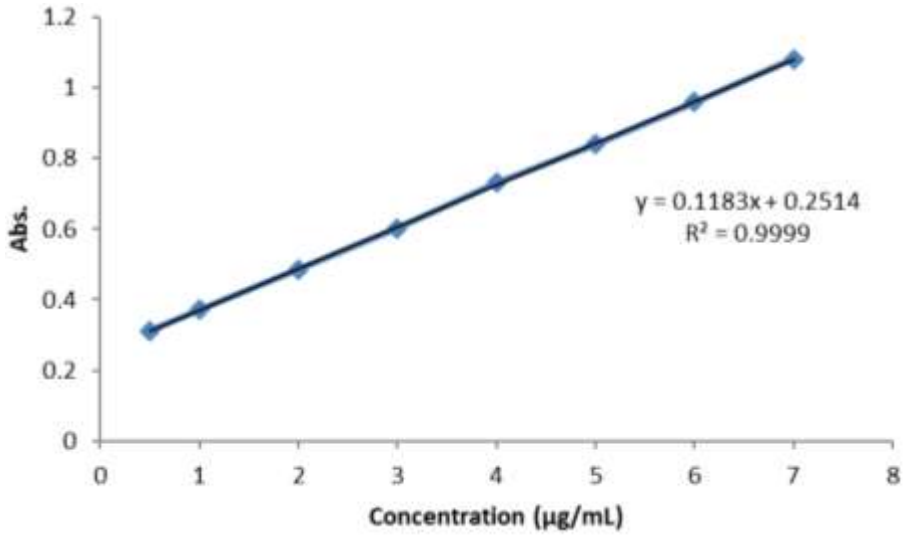

Figure 11. Calibration curve of rutin reacted with Ag-NPs.

Table 2. Spectral data for determination of diosmin and rutin through silver nanoparticles formation.

Parameter	Diosmin	Rutin
Linearity range (µg/mL)	1.0-8.0	0.5-7.0
Limit of detection LOD (µg/mL)	0.26	0.12
Limit of quantitation LOQ (µg/mL)	0.78	0.36
Slope (b)	0.1139	0.1183
Intercept (a)	0.0152	0.2514
Correlation coefficient (r)	0.9994	0.9999

Calculated on the basis of the molecular weight of the drug A= a+bc.

Table 3. Determination of authentic diosmin and rutin through silver nanoparticles formation.

Parameter	Diosmin		Rutin	
	Taken (µg/mL)	Recovery %	Taken (µg/mL)	Recovery %
	1	99.65	0.5	100.54
	2	99.76	1	100.57
	3	100.43	2	99.16
	4	99.20	3	99.28
	5	100.87	4	99.95
	6	99.78	5	100.00
	7	100.65	6	99.65
	8	100.09	7	99.49
Statistics				
Mean		100.-5		99.83
N		8		8
V		0.50		o.44
±S.D.		0.71		0.66
R.S.D.		0.71		0.66

*Mean of three different experiments.

Table 4. Determination of fractionated diosmin and rutin through silver nanoparticles formation.

Parameter	Diosmin		Rutin	
	Taken (µg/mL)	Recovery %	Taken (µg/mL)	Recovery %
	4	96.43	3	95.11
	5	97.00	4	96.35
	6	96.21	5	96.32
	7	95.01	6	95.71
	8	95.01	7	94.98
Statistics				
Mean		95.93		95.69
N		5		5
V		0.74		0.52
±S.D.		0.86		0.72
R.S.D.		0.90		0.75

*Mean of three different experiments.

Table 5. Determination of diosmin and rutin in their pharmaceutical formulations.

Parameter	Dioven® Tablets (For Diosmin)		Ruta C60® Tablets (for rutin)	
	Taken (µg/mL)	Recovery %	Taken (µg/mL)	Recovery %
	1	99.65	0.5	100.54
	2	99.76	1	100.57
	3	100.43	2	99.16
	4	99.20	3	99.28
	5	100.87	4	99.95
	6	99.78	5	100.00
	7	100.65	6	99.65
	8	100.09	7	99.49
Statistics				
Mean		100.05		99.83
N		8		8
V		0.46		0.44
±S.D.		0.68		0.66
R.S.D.		0.68		0.66

*Mean of three different experiments.

size around 30 nm (Figure 12).

Haemostatic activity

The total extract of *P. salicifolia* seeds significantly (P≤0.001) decreased bleeding time and amount of bleeding when administered orally to rats at doses of 10 mg/kg while the rest of cited drugs exhibited more significant hemostatic activity (P≤0.0001) (Table 9). The percent reduction in the bleeding time of the authentic diosmin, authentic rutin, total extract, fractionated diosmin, fractionated rutin, diosmin dosage form and rutin dosage were, 12.08 to 36.06 and 19.09 to 42.32% for the cited drugs and their nanoparticulated forms respectively while their percent reduction in amount of bleeding were as follows: 12.30 to 39.04 and 16.00 to 46.15%.

Conclusion

The gained results supported the advantage of the use of PVP-craped AgNPs as colorimetric probe for determination of diosmin and rutin in *P. salicifolia* seeds'

Table 6. Application of standard addition technique for determination diosmin and rutin in their pharmaceutical formulations.

Parameter	Dioven® Tablets			RutaC60® Tablets		
	Taken (µg/mL)	Added (µg/mL)	Recovery %	Taken (µg/mL)	Added (µg/mL)	Recovery %
	5	-	100.44	6	-	99.99
		5	99.62		6	99.57
		6	99.64		7	99.01
		7	98.68		8	99.50
Statistics						
Mean*			99.60			99.52
N			4			4
V			0.46			0.26
±S.D.			0.68			0.51
R.S.D.			0.68			0.51

*Mean of three different experiments.

Table 7. Statistical data for determination of diosmin and rutin through silver nanoparticles formation.

Parameter	Diosmin		Rutin	
	Suggested Method	Reported Method [55]	Suggested Method	Reported Method [56]
Statistics				
Mean	100.05	100.55	99.83	99.81
N	8	5	8	5
V	0.50	0.83	0.44	0.91
±S.D.	0.49		0.44	0.91
t (2.201)	0.39		0.50	
F (4.070)	1.69		2.07	

*Theoretical values of t and F at p =0.05.

Table 8. The intra-day and inter-day accuracy and precision data for determination of diosmin and rutin through silver nanoparticles formation.

Parameter	Intra-day				Inter-day			
	Taken (µg/mL)	Found (µg/mL)	Recovery %	RSD %	Taken (µg/mL)	Found (µg/mL)	Recovery %	RSD %
Diosmin	4	3.96	99.01	0.38	4	4.04	100.91	0.54
Rutin	4	3.94	98.55	0.61	4	3.96	99.01	0.84

*Mean of six different experiments.

extract, fractions, authentic and dosage form. The method used is economic, simple, rapid, does not require various elaborate treatment and environmentally safe, moreover, this method was successfully validated and exhibited great potential for determination of different samples with high accuracy and precision meanwhile *in vivo* haemostatic studies revealed that the extract and analyzed drugs exhibited significant haemostatic activity

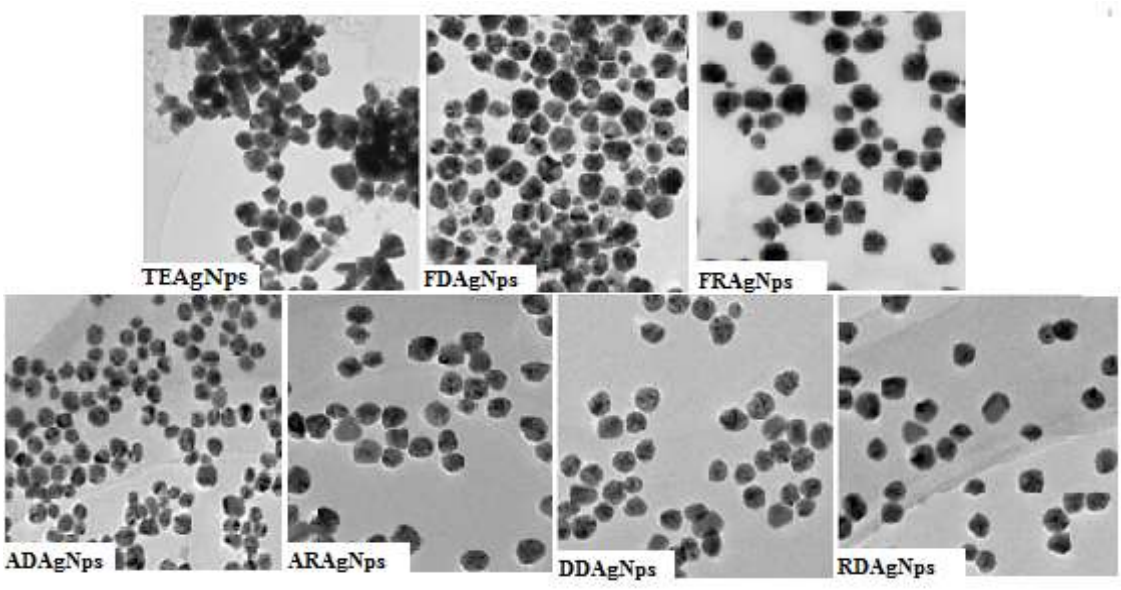

Figure 12. TEM micrograph of silver nanoparticles synthesized by using silver nanoparticulated cited drugs: the silver nanoparticles AgNps of Authentic Diosmin (AD), Authentic Rutin (AR), Total Extract (TE), Fractionated Diosmin (FD), Fractionated Rutin (FR), Dosage form Rutin (DR) and Dosage form Diosmin (DD).

Table 9. Effect of normal control, authentic diosmin, authentic rutin, total extract, fractionated diosmin, fractionated rutin, diosmin dosage form and rutin dosage form and their silver nanoparticulated forms on bleeding time and amount of bleeding in rats.

Group	Bleeding time (S)		Amount of bleeding (ml)	
	Drug	Drug AgNps	Drug	Drug AgNps
Normal control	147.51±6.05		0.3250±0.027	
Authentic Diosmin (AD)	94.29±4.30**	85.07±3.97**	0.1980±0.015**	0.1750±0.016**
Authentic Rutin (AR)	99.31±4.55**	94.53±4.95**	0.2195±0.016**	0.2140±0.015**
Total Extract (TE)	129.68±5.68*	119.35±5.25*	0.2850±0.021*	0.2730±0.017*
Fractionated Diosmin (FD)	96.50±3.95**	89.10±3.88**	0.2145±0.019**	0.2010±0.018**
Fractionated Rutin (FR)	110.59±4.62**	105.70±4.80**	0.2205±0.019**	0.2175±0.016**
Dosage form Diosmin (DD)	97.48±4.15**	92.65±3.95**	0.2015±0.019**	0.1830±0.017**
Dosage form Rutin (DR)	109.20±4.35**	101.30±4.20**	0.2185±0.018**	0.1935±0.017**

Dose (10 mg kg^{-1} b.wt.); Bleeding time; values are expressed as mean ± SEM (n=6); *P≤0.001, **P<0.0001 compared to control group; Amount of bleeding; Values are expressed as mean ± SEM (n=6); *P≤0.001 compared to control group.

which was manifested as deceased bleeding time and amount of bleeding when orally administered to rats.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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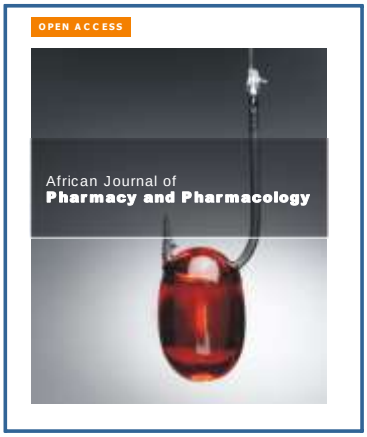

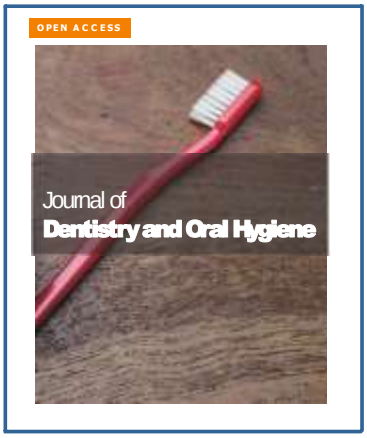

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