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QUANTIFICATION OF SINENSETIN IN EXTRACTS OF ORTHOSIPHON STAMINEUS USING HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

The present study aimed to develop a high performance thin layer chromatography method for the determination of sinensetin and apply it to standardize the extracts *Orthosiphon stamineus.* The samples and a series of standards were applied in duplicate on silica gel plate, which was then developed using mobile phase comprising chloroform and ethyl acetate (6:4, v/v) to a distance of 5 cm. Afterwards, the plate was dried and subjected to densitometry at 366 nm for the quantification of sinensetin. The limit of detection (LOD) and limit of quantification (LOQ) was found to be 0.05 and 0.50 µg/ml, respectively. The method showed linearity in a range of 0.50-100.00 µg/ml with correlation coefficient 0.9988. The recovery, intra- and inter day accuracy were found to be 95.67-97.65, 97.64-99.54 and 97.83-98.57%, respectively, with relative standard deviation (RSD) less than 5%. The extracts contained sinensetin from 0.470-1.335 mg/g/. The results of this study indicate that the developed method is sensitive, reliable, repeatable and reproducible and may be applied to standardize extracts of *Orthosiphon stamineus*..

Keywords: HPTLC, *Orthosiphon stamineus,* Sinensetin, Standardization

INTRODUCTION

Standardization of herbal products is always challenging due to their complexity, unknown nature of chemical constituents and inadequacy or unavailability of analytical methods as well as standards. In recent years, despite substantial growth in sales, herbal products are unable to get in the mainstream of pharmaceuticals due to inconsistency in quality and efficacy. Moreover, extracts of well defined constituents are the requirement of clinical trials. Therefore, it is imperative to develop analytical methods that can be used to standardize herbal products.

For quantitative analysis, marker compounds, characteristic to a plant, are required to be used as standards. Recently, Li *et al*. (2008) and Hussain *et al*. (2009) have summarized different categories of compounds that may be used as analytical markers to develop methods of analyses using the modern analytical tools. Keeping these points in view, the present study is undertaken to develop an analytical method using sinensetin as an analytical standard employing high performance thin-layer chromatography in combination with UV-densitometry.

Sinensetin, 5, 6, 7, 3', 4'-pentamethoxyflavone, is a pharmacologically active compound that can be used as an analytical marker/standard to develop analytical methods for quality control of herbal products containing this compound. Therefore, in the present study we have selected sinensetin to develop an analytical method to standardize extracts of a medicinally and commercially important plant, *O. stamineus* Benth. *(Lamiaceae).* The plant is distributed in tropical and sub-tropical region and is extensively used as a traditional medicine to treat various ailments in many countries of the world (Goh *et*

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al., 1994, Awale *et al.*, 2003a and 2003b). Tea (Java tea or Misai kuching tea) made from leaves of the plant is getting popular in many countries of the world due to its several therapeutic properties. The plant has also been investigated for pharmacological activities such as antioxidant, hepatoprotective, gastroprotective, antipyretic, antihypertensive and diuretic (Mariam *et al.,* 1996, Akowuah *et al.,* 2004a, Akowuah *et al.,* 2004b, Yam *et al.,* 2007, Arafat *et al.,* 2008, Yam *et al.,* 2009a, Yam *et al*., 2009b). Polyphenols, flavonoids, triterpenes and saponins are the main phyotochemicals reported in the plant. Among phenolic compounds, the plant has a significant amount of sinensetin, hence in the present study we have selected this compound as a marker to standardize extracts of the plant.

Various high performance liquid chromatography (HPLC) methods with UV detection have been reported for the quantification of sinensetin (Akowuah *et al*., 2004a, Loon *et al.,* 2005, Wang *et al.,* 2008). Ahmad *et al.* (2008) have also reported the quantification of sinensetin using disposable array sensors. As per our literature review, two short communications were reported about the quantification of sinensetin using HPTLC, first by Hossain *et al.* (2004) and second by Hossain and Salehuddin (2009) for the simultaneous quantification of sinensetin and tetramethoxyflavone. Akowuah *et al.* (2004) have also analyzed extracts of *O. stamineus* using HPTLC to find the content of certain compounds in extracts including sinensetin. To date, there is no report describing the complete development and validation of a method for the quantification of sinensetin employing HPTLC in combination with UV-densitometry. Therefore, the present study is aimed to describe for the first time a complete development and validation of a method for the quantification of sinensetin using HPTLC in combination with UV-densitometry, and its successful application to standardize extracts of *O. stamineus.*

EXPERIMENTAL

Plant material

Seven extracts of *O. stamineus* labeled as UITM 6 (50% methanol), UITM2 (ethanol), UITM (50% acetone), NHSIDE05 (aqueous), NHSIDE06 (aqueous), HMV06 (aqueous), HVM07 (aqueous) and CEPP01 (aqueous) were obtained from the Pilot Plant, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia.

Four batches of air dried leaves of the plant having code numbers J0701, J0702, J0703 and J0704 were taken from the Herbal Secretariat, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia. The leaves were pulverized and extracted with methanol for 1.5 h at 40°C. The extracts were filtered and dried in *vacuo* at 40°C.

Chemicals

Analytical grade chemicals procured from Merck included ethyl acetate, chloroform, methanol, ethanol, acetone and silica gel $60F_{254}$ plates of different sizes. Sinensetin of more than 98% purity was purchased from Sigma Aldrich.

Instrumentation

The analysis was performed on HPTLC system of CAMAG (Berlin, Germany) comprising densitometer (CAMAG Model-3 TLC Scanner) equipped with winCATS 4 Software, semi-automatic sampler (Linomat-5) and image recorder (CAMAG PROSTER 3).

Preparation of standard solutions

A stock solution of sinensetin was prepared in methanol to a concentration of 1 mg/ml, whilst a series of working standard solutions of concentrations 0.50-600 µg/ml were prepared by diluting the stock solution with methanol.

Development and validation of the method

The method was developed and validated by applying the standard solution on HPTLC plate (5 x 10 cm) as: three tracks, band length 6 mm, distance from lower edge of the plate 8 mm and the first application at 15 mm from left edge of the plate. The plate was developed in horizontal Teflon DS chamber and vertical twin troughs using various mixtures of solvents. Both types of chambers were allowed to saturate for 10 min before starting the flow of mobile phase.

The lowest limit of detection (LOD) was determined at signal to noise ratio (S/N) of 3 : 1 by application of a series of 2-fold dilutions of the standard solution, whereas the lowest limit of quantification (LOQ) was taken at S/N of 10 : 1. The linearity of the method was evaluated over the whole range of samples investigated by constructing calibration curves between concentration and peak area/peak height. Efficiency of the assay was evaluated by applying a known concentration of the standard solution as a control.

Three concentrations of the standard solution, 30, 60 and 100 µg/ml, were used to evaluate recovery, intraday and inter-day accuracy and precision of the method. For recovery, 200 mg of powder of leaves of the plant was spiked with the standard solutions and extracted in the same way as mentioned in the preparation of methanol extracts. An un-spiked sample was also extracted in the same way as that of the spiked sample, which served as a

control. The percentage recovery of true value of the analyte was determined by comparing the content of sinensetin of the spiked samples to that of the un-spiked samples.

To evaluate repeatability, intraday accuracy and precision, and reproducibility, inter-day accuracy and precision, three concentrations of the standard solution mentioned above were applied on TLC plate and the plate was developed by solvent system comprising chloroform and ethyl acetate (6:4, v/v) and scanned at 366 nm six times in a single day for intraday accuracy and precision, and once daily for 6 consecutive days for inter-day accuracy and precision. The accuracy was evaluated by quantifying the applied concentrations from the calibration curves of concentration versus peak area or peak height and precision was evaluated by relative standard deviation (RSD).

The plates were developed to various distances to find out the optimum distance that gave suitable separation without effecting peaks symmetry and band broadening. Samples were applied on the plate by band-wise and spot-wise to find out the suitable way of application. The robustness of the method was evaluated by small deliberate changes in mobile phase proportions, saturation time, distance of development and scanning wavelengths. The plates were also sprayed with Natural Product/Polyethylene Glycol (NP/PEG) reagent and the results were compared to that of the plates without application of the reagent.

Preparation of samples

The stock solution of aqueous extracts having a concentration of 100 mg/ml was prepared in a mixture of methanol and water (1:1, v/v). The working sample solutions of concentration 50 mg/ml were prepared by diluting the stock solution in methanol.

The stock solutions of all the other extracts were prepared to a concentration of 50 mg/ml in methanol, whereas working sample solutions of concentration of 5 mg/ml were prepared by diluting the stock solution in methanol.

Application of samples and standards

One plate was prepared for aqueous extracts and one for other extracts. For the former, samples and the standards were applied band-wise on pre-coated TLC plate (20 X 10 cm) as: 19 tracks, application volume 4 µl, 8 mm from lower edge, 15 mm on either side of the plate and distance between bands 9.4 mm. The standard solution was applied in a volume of 2, 4, 8, 12 and 20 µl per application that was equivalent to 0.1, 0.2, 0.4, 0.6 and 1.0 µg. For the later, the extracts were applied as stated above, whilst the standard solution was applied in volume of 2, 4, 8, 12 and 20 µl per band that was equivalent to 0.05, 0.1, 0.2, 0.3 and 0.4 µg, respectively. On this plate a known concentration of the standard solution equivalent to 0.25 µg was applied as a control. Except the control all the applications were in duplicate on both the plates.

Chromatographic development and documentation

The plates were developed in saturated horizontal DS Teflon chamber with solvent system comprising chloroform and ethyl acetate (6:4, v/v) and the plates were allowed to develop to a distance of 5 cm from the lower edge. The plates were dried with a gentle stream of warm air and scanned at 366 nm, and the quantification of sinensetin was carried out by winCATS Software using linear regression. Bands corresponding to peaks of the sample and the standards were assigned and scanned in a range of 400-200 nm for peak purity. Finally, images of the plates were documented at 254 and 366 nm.

The amount of sinensetin in the sample was calculated by the following formula and the results were expressed as mg/g of extract.

$$Q = VC / W$$

Where Q is the amount of sinensetin in the test sample, C is the concentration of sinensetin which is calculated from a calibration curve, V is the final volume of extracts (ml) and W is the weight of the test sample in grams.

Statistical analysis

All the samples and the standards were analyzed in duplicate and the results were presented as mean ±SD. For recovery studies each sample was extracted thrice and then each sample was analyzed in triplicate. For accuracy studies each of the samples was applied in triplicate and scanned six times and results were averaged. Recovery and accuracy was evaluated by % age of true value of the analyte added and recovered, whereas precision was evaluated by RSD.

RESULTS AND DISCUSSION

The development of the method was started by observing the spots of sinensetin on HPTLC plate in UV chamber at two fixed wavelengths, 254 and 365 nm. It was found that at 366 nm sinensetin exhibited a characteristic bluish appearance. Therefore, this wavelength was used only for densitometry. Afterwards, various types of solvent systems were used to get optimum separation of the analyte from extracts of *O. stamineus*. It was noted that a solvent system comprising chloroform and ethyl acetate (6:4, v/v) had given optimum separation of the analyte from a complex matrix. Therefore, in the present study, the quantification of sinensetin was performed using this mobile phase. The plates were developed both in a

horizontal chamber (using pre-saturated and sandwich techniques) and vertical twin trough chambers. Here, it was noted that the development of plates in pre-saturated horizontal chamber had given better and fast separation of the analyte from a complex matrix, free from defects such as band broadening and smiling etc.

To optimize the suitable distance of plate development, plates having different strengths of the standard solution were developed with the selected mobile phase, and linearity was evaluated by plotting calibration curves between concentration and peak area and well as peak height. It was found that the optimum distance for the development of the plate was 5 cm from the lower edge. Furthermore, the linearity was compromised by developing plates to a distance of 6 cm or more. Therefore, in the present study, plates were developed at a distance of 5 cm.

On HPTLC plate, samples might be applied spot-wise or band-wise, therefore, sample application was also evaluated and it was found that band-wise application produced symmetrical peaks. These results suggested band-wise application in further experiments.

The plates were scanned at different wavelengths and it was found that the recovery of the analyte was better at 366 nm. We had also used the wavelength (219 nm) as stated by Hossain *et al.* (2005), and it was found that the scanning at 219 nm had not produced accurate results.

To enhance the sensitivity of the assay, the plates were sprayed with NP/PEG reagent and the results were compared to that of the plate without spray. It was observed that the application of the reagent did not enhance sensitivity of the method. Moreover, the application of the reagent had reduced the clarity of the plate. These results indicate that there it is no need of derivitization for the detection and quantification of sinensetin.

The LOD was found to be 0.05 µg/ml at S/N 3, whereas LOQ was taken to be 0.50 µg/ml at S/N 10. The LOQ value was confirmed by analyzing the concentration 6 times. The linearity of the method was found to be in a range of 0.50-100.00 µg/ml (R^2 = 0.9988), both by plotting concentration of the analyte versus peak area as well as peak height.

The results of recovery, accuracy and precision of the method are presented in Table I. These results indicated the values of recovery, intraday and inter-day accuracy were in a range of 90.97-97.69% with precision (RSD less than 5). It was clear from these results that the method was accurate and the results were not been compromised in intra- and inter-day analysis.

The Robustness of the method was evaluated by a slight change in mobile phase proportions, saturation time, developing distance of the plate and scanning wavelengths. It was found that accuracy of the method was not affected by such slight changes, hence, the method was found to be robust.

Table 1: Recovery, intraday and inter day accuracy and precision values of sinensetin by high performance thin-layer chromatography (HPTLC)

Concentration (µg/ml)	Recovery (n = 3)		Intraday (n = 6)		Inter-day (n = 6)	
	Mean (%)	RSD (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
30	95.67	3.24	97.64	3.76	97.83	4.24
60	95.80	3.76	98.98	4.02	98.24	3.79
100	97.69	2.99	98.54	4.38	98.57	3.48

RSD (relative standard deviation)

The validated method was applied successfully to quantify sinensetin in different types of extracts of leaves of *O. stamineus*. In aqueous extracts, the quantification of sinensetin was carried out from calibration curve of concentration versus peak area using linear regression equation, Y = 3080.788 + 353.052 X, having correlation coefficient (R^2 = 0.9922) and standard deviation (SD = 8.02), whereas, in the other extracts (methanol and acetone etc.) sinensetin was quantified using calibration curve of concentration versus peak area with linear regression equation, Y = 6712.312 + 301.568 X, having R^2 = 0.9894, and SD= 8.63. Efficiency of the method, determined from the recovery of the standard as a control, was found to be 96.46% (SD = 8.02) on the plate of aqueous extracts, and 95.50% (SD= 8.63) on the plate of other extracts. It indicated that the method was efficient. The method was applied to determine the amount of sinensetin in various types of extracts of the plant, the results of which were summarized in Table II. These results indicate that sinensetin is found in varying amount in all the extracts. 3-D densitograms of aqueous and methanol extracts, and the standard are presented in Figure 1. Image of the plate, documented at 366 nm, is given in Figure 2. These figures have shown the optimum separation of the standard from a complex

matrix such as extracts.

In UV-densitometry two peaks having the same retardation factor (R_f) need to be checked for peak purity that can be achieved from the color comparison of bands on image of the plate, and scanning the bands in UV/Visible range to compare the spectra of standard to that of the sample. In the present experiment, the peaks of the standard and corresponding peaks of the samples were scanned in UV range of 400-200 nm. The resulting spectra were compared to establish the identity. The comparison of the spectra given in Figure 3, indicated the peak purity. The similarity of color of the bands of the standard and the corresponding band in the extracts shown in the Figure 2, further confirms the peak purity.

Table II: The content of sinensetin in different extracts of *O. stamineus* by HPTLC (n=2)

Extracts	Sinensetin in extracts		Sinensetin in powdered leaves	
	mg/g	± SD	mg/g	± SD
Methanol J0701	1.335	0.0212	0.054	0.0212
Methanol J0702	1.280	0.0141	0.043	0.0141
Methanol J0703	0.990	0.0142	0.044	0.0142
Methanol J0704	0.825	0.0353	0.032	0.0353
UITM6 (50% Methanol)	0.820	0.0283	……..	……..
UITM 2(Ethanol)	1.205	0.0071	……..	……..
UITM (50% acetone)	1.300	0.0142	……..	……..
NHSIDE 05 (Aqueous)	1.015	0.0212	……..	……..
NHSIDE 06 (Aqueous)	1.250	0.0142	……..	……..
HVM 06 (Aqueous)	1.345	0.0212	……..	……..
HVM 07 (Aqueous)	1.125	0.0071	……..	……..
CEPP 01 (Aqueous)	0.470	0.0142	……..	……..
CEPP 02 (Aqueous)	1.140	0.0145	……..	……..

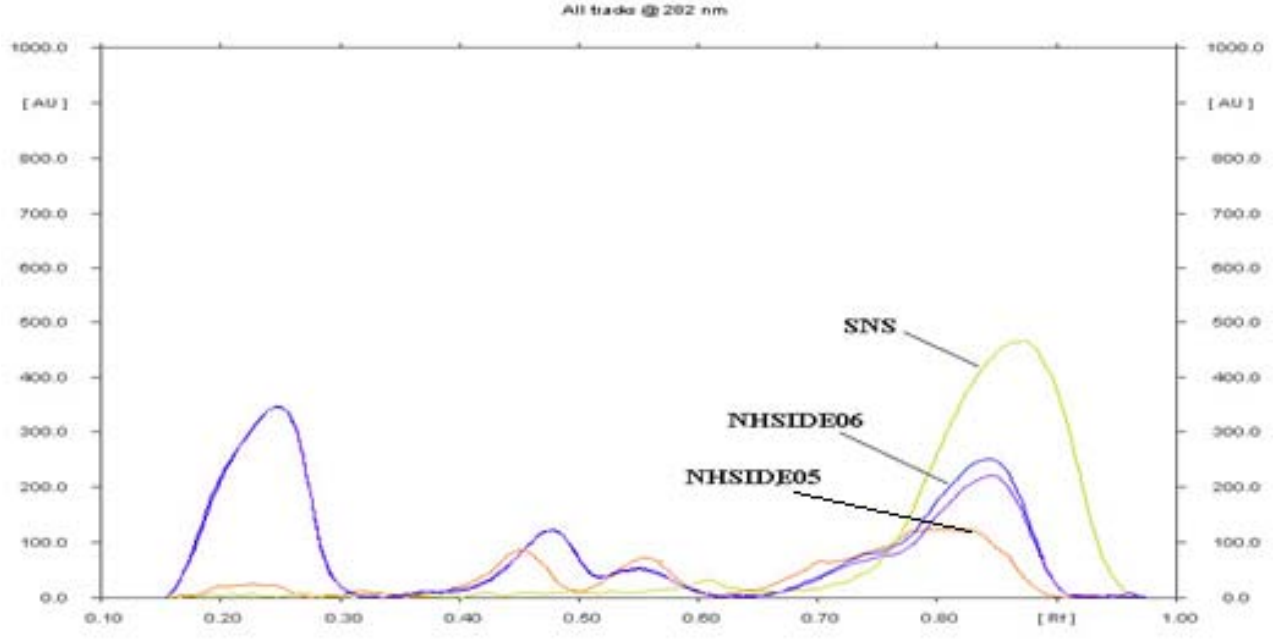

Figure 1: Three dimentional densitograms of sinensetin (standard) and extracts of *O. stamineus,* A (3D densitograms of aqueous extracts, NHSIDE 05 and 06 and sinensetin)

CONCLUSION

The results of the present study indicate that the method is sensitive, accurate and robust Moreover, the method is easy to perform and needs less solvents and time for the analysis. This method may be applied to standardize extracts and herbal products made from *O. stamineus*

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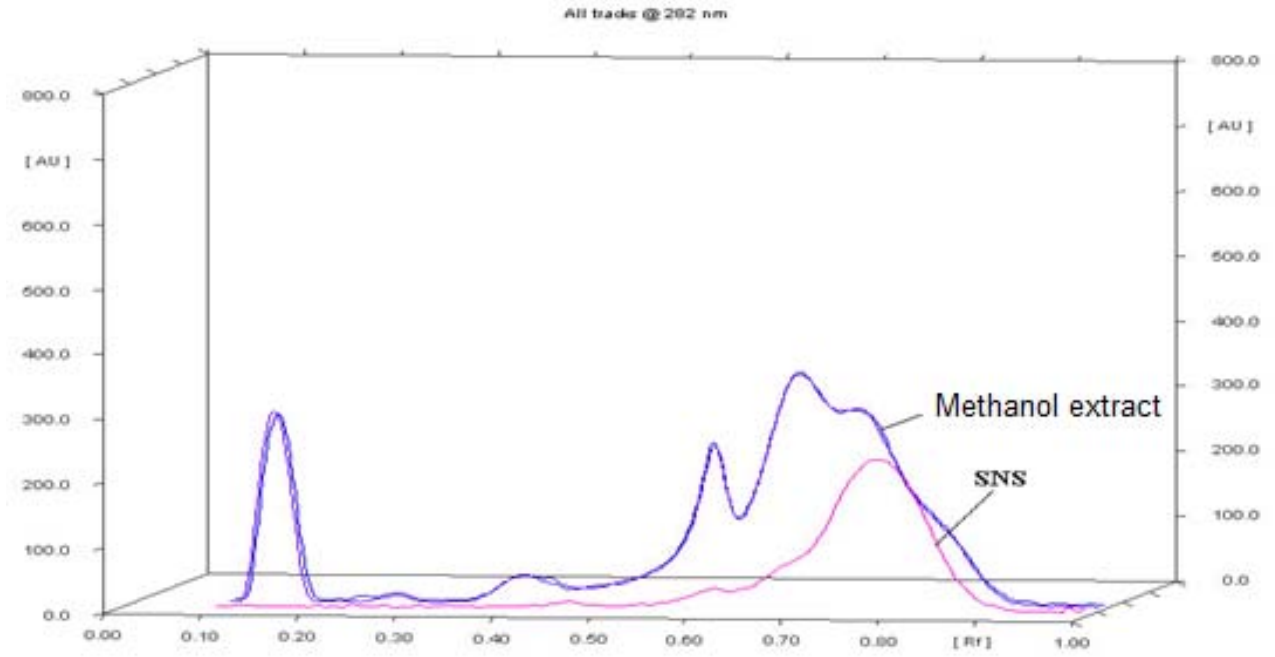

3D densitograms of methanol extract and sinensetin), SNS (sinensetin)

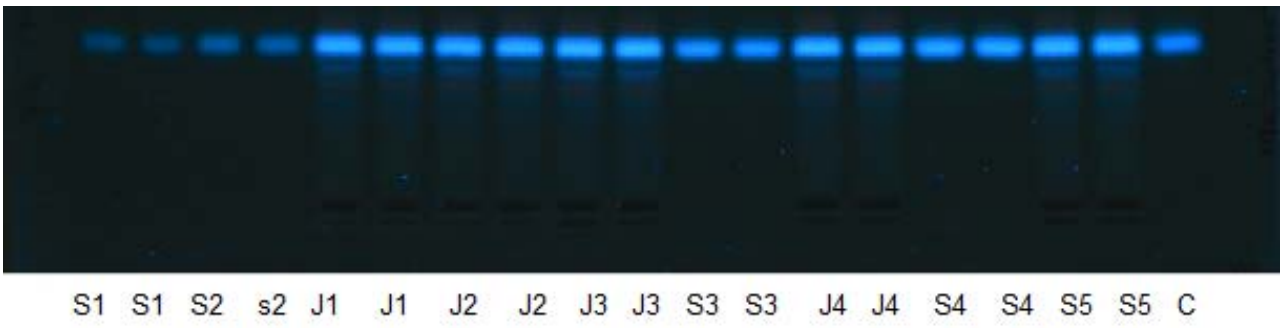

Figure 2: Image of HPTLC plate having a series of standards and various types of extracts of *O. stamineus* documented at 366 nm, S1-S5 (Different strengths of sinensitin as a standard), J1 (methanol extract J0107), J2 (methanol extract J0207), J3 (methanol extract J0307), J4 (methanol extract J0407), C (known cocentration of the standard as a control)

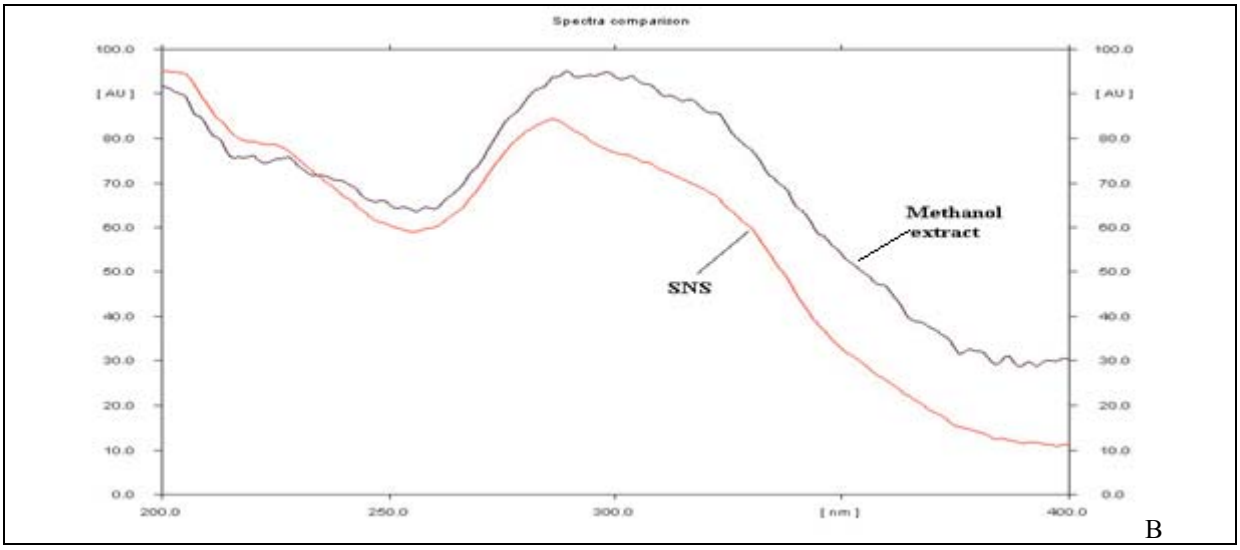

Figure 3: Spectra of sinensetin (SNS) as a standard and extract of *O. stamineus* in a range of 400-200 nm, A (SNS and aqueous extract), B (SNS and methanol extract)

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