CYTOTOXICITY EVALUATION AND CHARACTERIZATION OF CHLOROFORM EXTRACT OF LEAF OF *Piper sarmentosum* POSSESSING ANTIANGIOGENIC ACTIVITY

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Summary

The chloroform extract of leaves of *Piper sarmentosum,* a traditional medicinal plant, have shown promising antiangiogenic activity, IC_{50} 45 µg/ml. Therefore, present study aimed to investigate the extract to rule out the probability of the involvement of cell death in the activity, and characterize the extract using colorimetry, gas chromatography time of the flight mass spectrometry (GC-TOFMS) and high performance liquid chromatography (HPLC). Different concentrations of the extract were evaluated by MTT cell-viability assay using human hepatic carcinoma cell line (HepG2) and human umbilical vascular endothelial cell line (HUVEC). Vincristine sulphate (IC_{50} 0.016 µg/ml) was used as a positive control. The extract exhibited IC_{50} 76.24 µg/ml for HepG2 cells and 64.43 µg/ml for HUVEC. These IC_{50} values were found to be higher than IC_{50} (45 µg/ml) of the extract for antiangiogenic activity. The extract was analyzed for total amide content using colorimetric method and to characterize two main peaks by GC-TOFMS which were found to be pellitorine and sarmentine. In HPLC analysis, the extract was found to have pellitorine (0.020 mg/g) and sarmentine (0.006 mg/g). Both of these markers exhibited 30 % antiangiogenic activity. The results of this study indicate that the extract inhibits angiogenesis without changing morphology and viability of vascular endothelial cells.

Keywords: *Piper sarmentosum,* Cytotoxicity, MTT cell-viability, Antiangiogenesis, Amide content, Pellitorine, Sarmentine

Introduction

Piper sarmentosum Roxb. (*Piperaceae*) is cultivated as well as found wildly growing under shady trees in South East Asian region. The plant is popular due to its culinary and medicinal properties, and is available round the year. Phytochemically it

contains constituents such as amides, pyrones, flavonoids, sterols and neolignans [1, 2, 3].

As a traditional remedy, root and fruit of the plant are used to cure dysentery while root in combination with table salt is used to relieve toothache [4]. The root is believed to be effective in enurea, cough, flue, pleurisy and lumbago [5, 6, 7]. The plant is consumed as a carminative and drink of its various parts is used to relieve symptoms of malaria. In Thailand, the fruit is used as an expectorant [7, 8]. The plant has been investigated for a number of pharmacological activities such as anti-amoebic [9], antibacterial [10], anti-neoplastic [7], neuromuscular blocking [11], hypoglycemic [12], anti-malarial [13], antioxidant [14, 15, 16] and anti-TB [17, 18]. In our previous work, chloroform extract of leaves has exhibited significant antiangiogenic activities with IC_{50} 45 µg/ml [19]. In another study, ethanolic extract of the plant has shown anticancer properties on HepG2 cell IC_{50} 12.5 µg/ml and non-malignant Chang's liver cells 30 µg/ml [20].

Agent(s) having antiangiogenic properties should not be cytotoxic. Hence, antiangiogenic activity should be at lower concentration than that of cytotoxicity. Cytotoxic substances can affect morphology or viability of the cells. Cytotoxicity studies help to rule out the possibility of the involvement of cell death in antiangiogenesis. Therefore, it is important to evaluate antiangiogenic compounds for cytotoxicity using suitable cell lines. HUVEC cell line is important because vascular endothelial cells are involved in neo-vascularization while HepG2 is important to know whether cancer cells die directly or through inhibition of angiogenesis. Cytotoxicity activity can be measured by a number of methods such as MTT (microplate tetrazolium) assay, trypan blue assay, sulforhodamine B (SRB) assay, WST assay and clonogenic assay. In present study, MTT assay has been used because it is an easy and commonly used method.

Keeping it in view, present study was undertaken to evaluate an extract of leaves of *Piper sarmentosum* having significant antiangiogenic activity for cytotoxicity using two types of cell lines, malignant (HepG2) and normal (HUVEC), and to characterize the extract using colorimetry, GC-TOFMS and HPLC.

Materials and methods

Plant material and extraction:

Piper sarmentosum was collected in the month of March from Balik Pulau, Pinang, Malaysia and authenticated by Prof. Dr. Zhari Ismail, Herbal Secretariat, School of Pharmaceutical Sciences, Universiti Sains Malaysia, where a voucher specimen was deposited vide reference number 0071/06. The leaves were separated, dried at 40 ^{o}C and pulverized.

Leaf powder (150 g) was extracted sequentially using petroleum ether (2 L), and chloroform (2 L). The chloroform extracts were dried in vacuo at 40 ^{o}C.

Chemicals, solvents and cell lines:

Analytical grade/HPLC grade petroleum ether, chloroform, methanol and acetonitrile were procured from Merck. Distilled water was taken from the Main Chemistry Laboratory of the School of Pharmaceutical Sciences, Universiti Sains Malaysia. Chemicals purchased from Sigma Aldrich included DMSO, MTT [(3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], penicillin, streptomycin, heat inactivated fetal bovine serum (HIFBS), suramin sodium, fungizone, glutamine, gentamycin, α-aminocaproic acid, PBS, glycine, NaCl, piperine, and vincristine sulphate. Microtitre plates of 96-well were purchased from Corning Incorporated, USA. Human hepatic carcinoma cell line (HepG2, ATCC# HB 8065) and human umbilical vascular endothelial cells (HUVEC-CS, ATCC # CRL 2873) were used.

Instruments:

Plates for cytotoxicity were evaluated by Microplate reader (BioTek, USA). The colorimetry was performed using UV/visible spectrophotometer (PerkinElmer Lambda 45, Shelton, CT, USA). Liquid chromatography was performed on HPLC system 1100 series of Agilent Technologies, Waldronn, Germany, equipped with degasser (G1379 A), quaternary pump (G1311 A), auto sampler (G1313 A), column oven (G1316 A) and UV detector (G 1314 A).

Preparation of sample and standards for MTT cell-viability assay:

Stock solution of chloroform extract of leaves was prepared to a concentration of 1 mg/ml with methanol. The working samples solutions were prepared by further diluting the stock solution with methanol as: 0.50-200.00 µg/ml for HepG2 cell line, and 3.12 – 200.00 µg/ml for HUVEC cell line.

Stock solution of vincristine sulphate was prepared in distilled water to a concentration of 1 mg/ml. The working standard solutions were prepared by diluting the stock solution with methanol to a concentration of 0.10-200.00 µg/ml.

Preparation of Medias:

For HepG2 cell line, MEM medium (Gibco, UK) was prepared by adding HIFBS (10% w/w of medium) and penicillin and streptomycin (each 1% w/w of medium).

For HUVEC cell line, MEG-2 medium (Singapore) was prepared by adding HIFBS (10% w/w of medium) and penicillin and streptomycin (each 1% w/w of medium).

MTT cell-viability assay:

Cells were diluted to a density of 10 X 10^3 cells/ml in the culture medium. Then 150 µl of the cell culture was added in the wells of a 96-well microtitre plate. The plate was incubated at 5% CO_2, 37 ^oC and 95% humidity for 24 h. Each concentration of the sample or standard (100 µl) was added in the wells in 4 replicas. Twenty micro litters of MTT (5 mg/ml in PBS glycine buffer of pH 10.5, which was prepared by 0.1 M glycine and 0.1 M NaCl) was added in each well and the plate was placed on shaking table for 5 min in order to mix the cells with the sample and the MTT. Then the plate was incubated at 37 ^oC and after 4 h media and MTT was aspirated from the wells. Then 200 µl of DMSO and 25 µl of glycine buffer were added in each well to dissolve the residue, formazan a metabolite of MTT. The optical density of the residue was measured at 560 nm and background at 670 nm was subtracted. Four wells having all components except the sample served as a control. Percentage inhibition was calculated using the following equation:

$$\text{Viability} = As - Ab \,/\, Ac - Ab$$
$$\text{Inhibition} = (1 - \text{Viability}) \times 100$$

Where As is absorbance of the sample, Ab absorbance of the blank and Ac is absorbance of the control.

Characterization of the extract:

Estimation of total amides [21]- Hundred micro liters of alcoholic sample solution (5 mg/ml) or standard solution (different concentrations), concentrated sulfuric acid (2 µl) and 5% gallic acid solution in methanol (100 µl) were mixed in a test tube. Then the mixture was heated in water bath for 10 min, and absorbance was measured at 660 nm against a blank containing equivalent amount of methanol in place of the sample. Piperine solutions in a range 0.10-100.00 µg/ml were used as a standard and amide content was calculated from the calibration curve applying linear regression. Total amide content in the extract was determined using the following equation and expressed as mg equivalents of piperine.

$$\text{Total amides} = (C \times V)/W$$

Where C (µg/ml) is concentration of piperine equivalents obtained from the calibration curve, V is the final volume of the extract in ml and W is the weight of extract in grams.

Characterization of main peaks of the extract by GC-TOFMS:

From HPLC chromatogram of the extract, two main peaks having retention time 4.1 and 4.5 min were selected to be characterized by GC-TOFMS. GC-TOFMS was performed at chromatographic conditions: column HP-5 (30 m X 0.32 mm ID, 0.25 Vm phase film), carrier gas (helium) at constant flow rate of 1.2 ml/min, temperature of the transfer line 250 ^oC, temperature of ion source 250 ^oC, temperature of injector 250 ^oC,

temperature of the oven kept 80 ^{o}C for 5 min and then raised to 280 ^{o}C at a rate of 7 ^{o}C / min, voltage of the detector 1650 V, sample volume 1.0 µl as split-less injection and data acquisition at 10 spectra/second (35-550 amu) with LECO Chromatof software. These peaks were found to be pellitorine (4.1 min) and sarmentine (4.5 min), which were used as markers to analyze the extract.

Quantification of markers in extract by high performance liquid chromatography:

Preparation of samples and standards- Stock solutions of pellitorine and sarmentine were prepared in methanol to a concentration of 50 µg/ml. A series of working standard solutions (0.01, 0.1, 0.5, 1.0 and 1.5 µg/ml) were prepared by further diluting the stock solution with mobile phase.

The working solution of extract was prepared in methanol to a concentration of 2 mg/ml. All the working solutions of the sample and the standards were filtered by 0.45 µm PTFE syringe filter (Whatman, Maidstone, England).

Chromatographic conditions and analysis- The sample and the standard solutions were analyzed in triplicate. Isocratic elution of the sample/standard solution (15 µl) was carried out with mobile phase comprising of methanol : water: acetonitrile (80: 15: 5 v/v). The elution time was 15 min at flow rate 1 ml/min and detection at 260 nm. The column (Hiber Rt 250-4, LiChrosorb RP 18, 10µm, Agilent Technologies) was maintained at 25 ^{o}C and the detector was operated in a sensitivity range of 0.005 AUFS with output of 15 mV. The data acquisition was performed by ChemStation version A. 08.03 and the markers were quantified using the external standard method.

Evaluation of the markers for antiangiogenic activity:

Animals and tissue preparation- The study protocol was approved by the Animal Ethical Committee of the School of Pharmaceutical Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia, vide letter number USM/PPSF/50 (011) Jld.2. Adult male *Sprague Dawley* rats of age 8-10 week were obtained from the animal house of the Universiti Sains Malaysia and kept in the animal holding to acclimatize. Standard pellet diet (Gold Coin, Penang) and water was supplied *ad libitum*. Animals were sacrificed by stunning and cervical dislocation. Then the animals were excised to get thoracic aorta and pieces of aorta were kept in 1 X PBS. After removing adipose tissue, aorta was cut into 1.0-1.5 mm thick cross sections.

Preparation of media- Growth media M 119 was prepared by dissolving 20 g heat inactivated fetal bovine serum (HIFBS) in 100 ml sterile water containing fungizone (1%), glutamine (1%), gentamycin (0.6%) and α-aminocaproic acid (0.1%).

Preparation of samples and antiangiogenic studies- The solutions of the pellitorine and sarmentine were prepared in DMSO to a concentration 50 µg/ml growth media M 199.

Antiangiogenic assay (rat aorta model) - The assay was performed by a method described by Adam *et al.* [22] with some modifications, briefly described as 0.5 ml fibrinogen (3 mg/ml in M 119) and 2.5 µl aprotinin (1 mg/ml in PBS) were transferred in each well of 48-well plate. Clean artery rings were rinsed in growth media and placed one in each well. After adding 15 µl thrombin (50 units /ml in 0.15 M sodium chloride) in each well, the plate was incubated for 1 h at 37 ^{o}C. Then 0.3 ml of the test sample was added in each well (6 replicate) while a solution of equivalent concentration without the sample was added in wells, which served as control. Suramin sodium in a concentration of 20 µg/ml was used as positive control. Equivalent amount of DMSO, used to prepare the sample, was also tested like the samples as negative control. The plate was then incubated at 37 ^{o}C for 5 days. The amount of new blood vessel growth was scored microscopically on day 5. Images were taken by microscope (OLYMPUS, Japan) equipped with digital camera (Leica DC 300) using Leica Qwin Pro V2.6 (Leica Imaging System, England). The percentage inhibition of angiogenesis was calculated using following equation.

$$\% \text{ Inhibition} = 100 - (\text{sample growth} / \text{control growth}) \text{ X } 100$$

Statistical Analysis:

Cytotoxicity studies are performed on four independent replicates and the results were averaged. For antiangiogenic activity the samples and the standard were evaluated in 6 replicate and the results were averaged. For characterization of the extract, samples were analyzed in triplicate and the results were averaged.

Results

Cytotoxicity studies:

In our previous studies, chloroform extract of the leaf of *Piper sarmentosum* exhibited antiangiogenic activity (100%) using rat aorta ring model [19]. Therefore, the work was extended to investigate the cytotoxicity of the extract using two types of cell lines, human hepatic carcinoma and human umbilical vascular endothelial cells.

The different concentrations of the extract viz 0.5, 1, 5, 10, 20, 50, 75, 100 and 200 µg/ml were evaluated for cytotoxicity on HepG2 using vincristine (IC_{50} 0.016 µg/ml) as positive control and the results of MTT cell-viability assay (Fig. 1) indicated that IC_{50} of the extract 76.24 µg/ml. The activity was calculated using linear regression $Y = 0.4116 X + 18.21$ obtained the plot of percentage inhibition versus concentration. These results indicated that the extract had higher IC_{50} for cytotoxicity as compared to the IC_{50} for antiangiogenesis.

Moreover, different concentrations of the extract viz 3.125, 6.25, 12.5, 25, 50, 75, 100 and 200 µg/ml were investigated for cytotoxicity on HUVEC and the results MTT cell-viability assay (Fig. 2) indicated IC_{50} of the extract 64.43 µg/ml. It was calculated by plotting the percentage inhibition versus concentration using linear regression equation (Y = 0.5243X + 17.264). These results were also quite similar to that of the HepG2 cells.

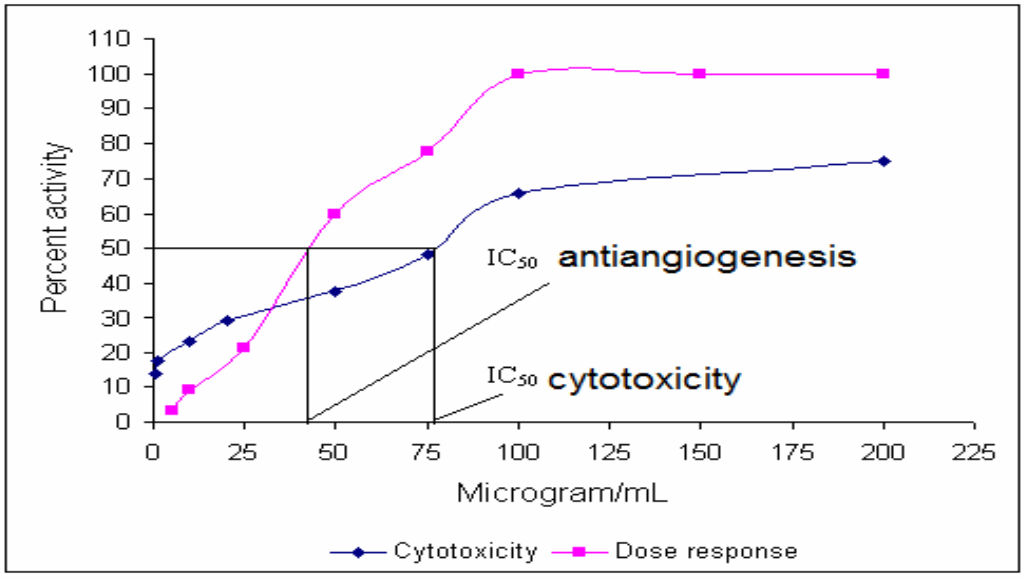

Figure 1 Plot of percent antiangiogenic activity versus concentration of chloroform extract of leaf of *Piper sarmentosum* as well as plot of percent inhibition of HEPG2 cells versus concentration of the extract

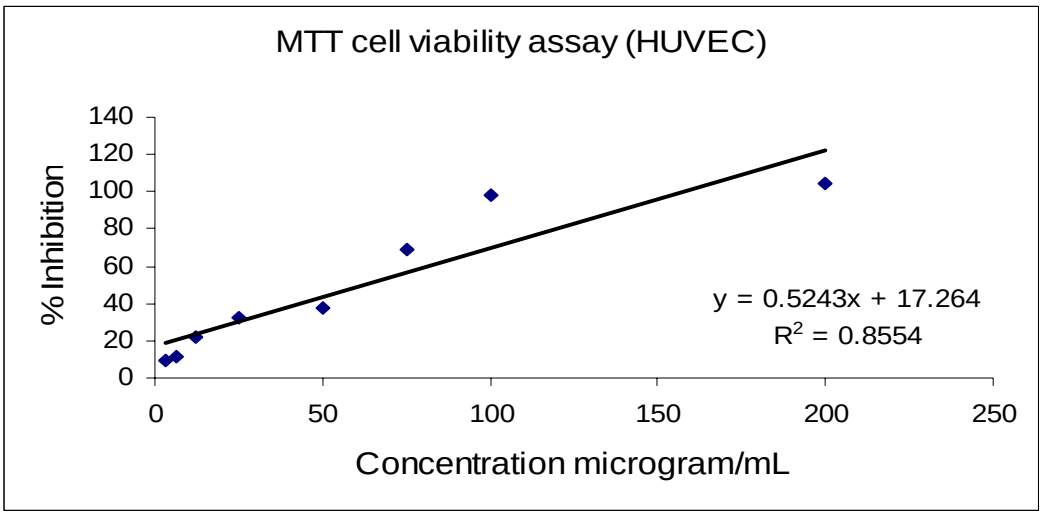

Figure 2 Plot of percent inhibition of HUVEC cells versus concentration of the leaf chloroform extract of *Piper sarmentosum*, HUVEC (human umbilical vascular endothelial cells)

Characterization of the extract:
Total amide content in the extract was calculated by linear regression equation Y = -8.90047e^3 + 6.680799 e^3, R^2 = 0.9963 and found to be 152.8 mg/g ± 3.96. The extract was analyzed by GC-TOFMS and the two main peaks were found to be pellitorine (molecular weight 223) and sarmentine (Molecular weight 221). The fragmentation patterns of these peaks are given in (Figure 3). These compounds were selected as markers to standardize the extract by HPLC. The pellitorine content determined by HPLC using linear regression equation, Y= 180.54 X + 6.9079, R^2 = 1, was 0.02 mg/g extract. The content of sarmentine determined by linear regression equation, Y= 846.2 X + 12.5760, R^2 = 1, was 0.0064 mg/g extract. The chromatograms of the extract and both the standards (Figure 4) indicated that the separation of the peaks was optimum.

Antiangiogenic activity of the markers:

A validated antiangiogenic assay was applied to evaluate the markers, pellitorine and sarmentine, for antiangiogenic activity. The activity of the markers was compared to suramin sodium, which served as positive control. It was found that both the markers exhibited 30% antiangiogenic activity while the positive control exhibited 100% activity. The activity of the chloroform extract of the leaf of *Piper sarmentosum* was higher as compared to the activity of both the markers. The activity of the extract was 50% at 45 µg/ml concentration (Figure 1). It indicated that other constituents of the extract also contribute in activity.

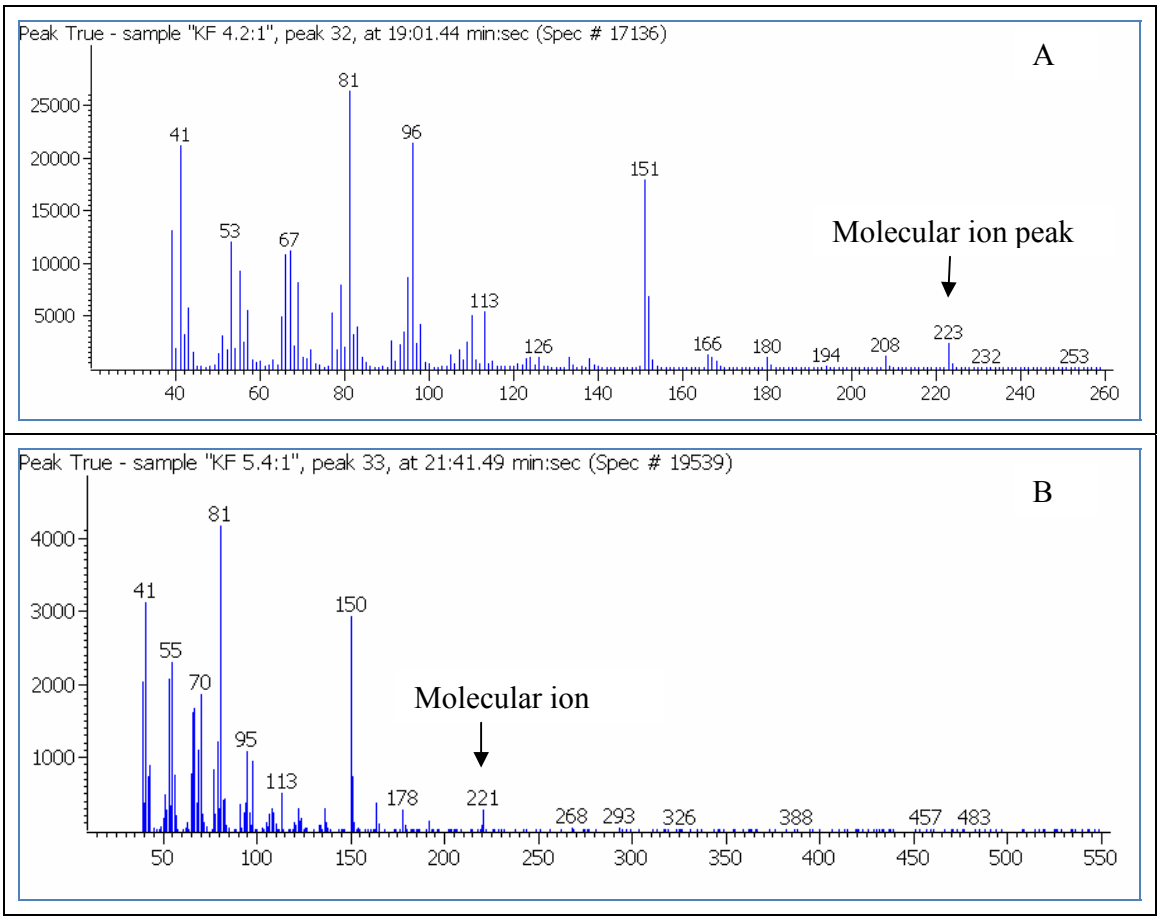

Figure 3 Gas chromatography time of the flight mass spectra of two main peaks of chloroform extract of the leaf of *Piper sarmentosum*, A (pellitorine); B (Sarmentine)

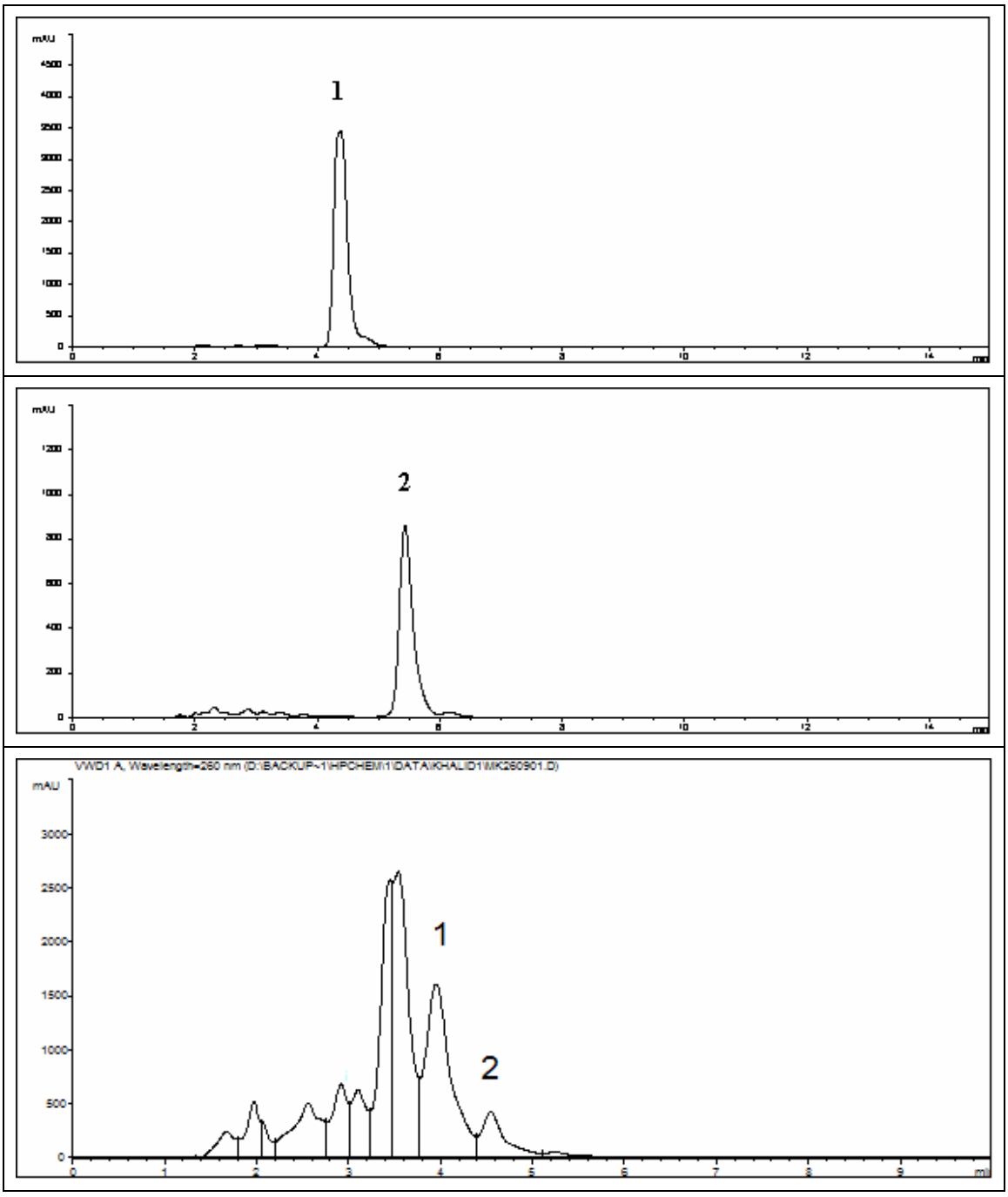

Figure 4 HPLC chromatograms of pellitorine, sarmentine and chloroform extract of leaf of *Piper sarmentosum* at 260 nm, 1 (pellitorine); 2 (sarmentine)

Discussion

Angiogenesis, formation of new blood vessels from the existing ones, is taking place in both healthy and disease states. Angiogenesis is initiated to supply nutrients and disposal of waste from the fast growing cells [23, 24]. Therefore, it was proposed that tumor growth and metastasis can be inhibited, if tumors are deprived of blood supply. Angiogenesis is a basic step in the progression of various diseases [25] and is controlled by complex chemical reactions through "on" switch (angiogenesis stimulators) or "off" switch (angiogenesis inhibitors) mechanisms [26, 27, 28, 29, 30]. Antiangiogenic agents are useful in treating diseases involving angiogenesis. These agents have special significance in cancer treatment because they act on genetically stable endothelial cells. Therefore, the chances of resistance may be less as compared to chemotherapeutic agents which act on genetically unstable cancer cells.

Cytotoxicity means toxic to cells and the chemical substances which can affect morphology and viability of cells are called cytotoxic. For *in vitro* cytotoxicity tests, cells are cultured under controlled conditions and substances are tested on these cells. The cells are treated with substances in various ratios and the effect on morphology and cell viability is investigated. These studies are used to calculate median inhibitory concentration/median lethal dose (IC_{50}/LD_{50}). The substance should be able to inhibit angiogenesis without killing vascular endothelial cells. Fast growing cells and certain conditions like tissue hypoxia stimulate vascular endothelial cells by different mechanisms to initiate angiogenesis. The vascular endothelial cells should be controlled for the inhibition of vascularization without killing them. Cytotoxicty studies help to rule out the possibility of the involvement of cell death in antiangiogenesis. Therefore, it is important to evaluate antiangiogenic compounds for cytotoxicity. In present study, MTT cell-viability assay was used to evaluate extracts for cytotoxicity.

MTT is a yellow water soluble dye which is reduced to formazan, a water insoluble compound, by living cells. The optical density of formazon is measured at 560 nm to calculate cell viability and inhibition of the cells. The comparison of spectra of treated and untreated cells gives the relative cell toxicity.

Chloroform extract of the leaf of *Piper sarmentosum,* which was obtained after extracting the leaves with petroleum ether has promising antiangiogenic activity and the activity is found to be taking place at lesser concentration than that required for cytotoxicity.

The extract was characterized for total amides because in our previous work correlation found between antiangiogenic activity and amide content. The extract was further analyzed by HPLC using two pharmacologically active analytical markers, pellitorine and sarmentine. The characterization of the extracts is important to standardize them to maintain claimed efficacy.

It is concluded from this study that chloroform extract of the leaves of *Piper sarmentosum* has antiangiogenic activity without killing vascular endothelial cells. The extract has also shown cytotoxicity on HEPG2 cell line at higher concentration as compared to the amount required for antiangiogenic activity.

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