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Full Length Research Paper

Antioxidant, antiangiogenic and vasorelaxant activities of methanolic extract of *Clerodendrum serratum* (Spreng.) leaves

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The *Clerodendrum serratum* (Spreng.) known as 'Timba Tasek' is widely used in Asian countries especially Malaysia as the traditional medicine to treat various diseases. This study aimed to evaluate the antioxidant, antiangiogenic and vasorelaxant activities as well as the chemical profiles of *C. serratum* leaves extract. The dried powder leaves of *C. serratum* were extracted serially with petroleum ether, chloroform, followed by methanol and water by maceration method. To elucidate the antiangiogenic properties, the inhibitory effects of these extracts on blood vessel growth formation were adapted in rat aortic ring assay. In another set of experiments, the possible vasorelaxant activity of *C. serratum* leaves extracts were examined on an isolated rat aortic ring preparations and responses of cumulative doses of noradrenaline (NA) were used. To determine antioxidant activity of this plant, the present study used well-established methods, that is, 2,2'-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging activity and trolox equivalent antioxidant capacity (TEAC) assay. The results showed that, amongst four extracts, methanolic extract of *C. serratum* (ME-CS) showed the most potent antioxidant, antiangiogenic and vasorelaxant activities. In another hand, qualitative study proved that ME-CS contains polyphenolics (hydrolysable tannins and flavonoids), terpenoids, saponins and may not contain any alkaloids. Therefore, while polyphenolics are the predominant compounds found in ME-CS, it is highly probable that they may play an important (dominant) role in antioxidant, antiangiogenic and vasorelaxant activity. Since all the three activities of *C. serratum* extracts end up in the same results, it is likely that, all the activities were contributed by same group (such as polyphenolics) or totally different group of chemical compounds that may act synergistically together with polyphenolics. Polyphenolics are responsible for antioxidant, antiangiogenic and vasorelaxant effects of plants as herbal therapy such as *C. serratum* leaves.

Key words: *Clerodendrum serratum* (Spreng.) leaves, polyphenolics, antioxidant, antiangiogenic, vasorelaxant, phytochemical analysis.

INTRODUCTION

The *Clerodendrum serratum* (Spreng.) locally known as 'Timba Tasek' is a plant belonging to Verbenaceae family. It has been well known among the people in central and South-east Asian Countries as well as the southern part of Africa. In Malaysia, this plant is consumed as a water decoction, to treat high blood pressure as alleged by its traditional use. Asmawi et al. (1989)

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also reported that water extract obtained from this plant, administered intravenously into anaesthetised rats, lowers the blood pressure.

Moreover, root extracts from *C. serratum* inhibits angiotensin converting enzyme (ACE), inflammation and prostaglandins synthesis (Nyman et al., 1998; Narayanan et al., 1999) as well proved to be antioxidant (Bhujbal et al., 2009). Apart from that, the presence of polyphenolics have been reported by (Sharma et al., 2000, 2009) in *C. serratum* extracts.

Plants have many phytochemicals which are potential source of natural antioxidants, such as phenolic diterpenes, flavonoids, tannins and phenolic acids (Dawidowicz et al., 2006) and also possess other biological properties.

Plant polyphenolics have been recognized to be a therapeutic target for cancer treatment and cardiovascular disease in the next decade (Yoysungnoen et al., 2008; Münzel et al., 2010). These benefits have been attributed to the presence of some polyphenolic compounds (Dell'Agli et al., 2004; Münzel et al., 2010), since the polyphenols enhance the production of vasorelaxant factors such as nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostacyclin, and inhibit both the synthesis of vasoconstrictor endothelin-1 in endothelial cells, and the expression of two major pro-angiogenic factors: vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2), in endothelium cells (Stoclet et al., 2004; Oak et al., 2005; Walter et al., 2009). Therefore, plant polyphenolics may exert a double-edged role of antiangiogenic and vasorelaxant activity, as well as have ability to prevent oxidant potentials of free radicals as natural source of antioxidants. There is no information on studies conducted investigating about antioxidant, antiangiogenic and vasorelaxant activities of the leaves of this plant.

For that purpose, it was evaluated that, the antioxidant, antiangiogenic and vasorelaxant activities of *C.serratum* leaves extracts has been locally consumed to treat hypertension (Asmawi et al., 1989), inflammation (Narayanan et al., 1999) and cancer. There is need for more scientific study which proves that it is traditionally used.

MATERIALS AND METHODS

Drugs and standards

The following reference chemicals namely: norepinephrine hydrochloride and verapamil hydrochloride were obtained from the Sigma-Aldrich, Germany. Chemicals used for making physiological salt solutions were: potassium chloride (Ajax Chem, Australia), potassium dihydrogen phosphate (GmbH, Germany), magnesium sulfate, calcium chloride, sodium bicarbonate, sodium chloride, glucose monohydrate (R & M Chem., UK), Fibrinogen (Calbiochem, USA), serum free M199 growth medium (Gibco®, USA), aprotinin, thrombin, ε-aminocaproic acid, L-glutamine, amphotricin B, gentamycin, bovine serum albumin plasma and Suramin were obtained from (Sigma-Aldrich, Germany) and Polyethylene glycol 400 (PEG-400) (Merck, Germany).

Experimental animals

All experimental procedures were approved by the Animal Ethics Committee of the School of Pharmaceutical Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia. The rats were maintained three per cage at constant temperature with a 12 h light/12 h dark photoperiod. Animals were allowed free access to food with standard laboratory chow, (Gold coin Sdn. Bhd., Malaysia) and tap water *ad libitum*. Adaptation period were allowed to acclimatize in the animal transit room for a minimum of one week before initiation of any experiment.

Plant material and extraction

The dried powder leaves of *C. serratum* (100 g) were macerated with solvents of increasing polarity from petroleum ether (a defatting step), chloroform , methanol and water respectively, in a flasks (250 ml) placed in a water bath with shaker model 903 (Brotech, Malaysia) at 45°C for 8 h. The extraction procedure with each solvent was repeated three times. The combined filtrate obtained was filtered using Whatman filter paper and then, the solvents were stripped off on a rotary evaporator (Büchi, Switzerland) under reduced pressure. The concentrated extracts were kept in a freezer at -70°C for 48 h and freeze-dried for 48 h.

Phytochemical screening

The active extract of *C. serratum* was screened for the presence of different classes of compounds by thin layer chromatography using silica gel G (Merck) plates of 0.25 mm thickness (Wagner et al., 1984). After development, the plates were sprayed with the following solvents and reagents for detection of the respective classes of compounds: anisaldehyde-sulphuric acid reagent heated for 5 min at 100°C (terpenoids), 10 ml of natural products-polyethylene glycol reagent (NP-PEG) and examined under UV 365 nm (Flavonoids) after which the Dragendorff's reagent (Alkaloids) (Wagner et al., 1984) was added.

Saponins were detected, by observing froth formation of the extract in a test tube after regular shaking which became stable approximately after 15 min (Wagner et al., 1984; Silva et al., 1998; Tona et al., 1998) and persisted on warming (Owoyele et al., 2008). In tannins, an aqueous solution of extracts that contains hydrolysible tannins are precipitated by 1 ml (10% w/v) of lead acetate and 1 ml (10% v/v) of acetic acid, while non-hydrolysible condensed tannins are soluble in 1 ml (10% v/v) of acetic acid (El Sissi and El Sherbeiny, 1967; Wagner et al., 1984).

Determination of polyphenolic contents

Total phenolic content

The total phenolic contents of *C. serratum* extracts were determined by using Folin-Ciocalteu reagent (Sigma Aldrich, Germany), according to the method reported by Slinkard and Singleton (1977), with gallic acid (3,4,5-trihydroxybenzoic acid) as standard. A solution of 2 mg/ml of extracts of *C. serratum* in 80% methanol and different concentrations of gallic acid (0.0312, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4 mg/ml in 80% methanol) were prepared. Briefly, 100 µl of different concentration of gallic acid solution and 100 µl of each extract were pipetted in different test tubes respectively, and 2 ml of distilled water was added into each test tube. Then, 200 µl of 2 N

Folin-Ciocalteu reagent was added into the respective test tubes. The contents were mix thoroughly and after 3 min, 1 ml of 15% (w/v) sodium bicarbonate solution ($NaHCO_3$) was added and the mixture was allowed to stand for 2 h with intermittent shaking at room temperature (24 to 26°C). Absorbance of the sam ple solutions (blue complex) was measured at 765 nm using Hitachi U-2000 spectrophotometer (Hitachi, Japan), against 80% methanol as a blank. The concentration of total phenolic compounds in the *C. serratum* extracts were determined and expressed as microgram of gallic acid equivalent, by using an equation which was obtained from standard gallic acid graph. The data were presented as mean ± SEM (n = 3).

Total flavonoid contents

The total flavonoid contents in *C. serratum* extracts were determined using aluminum chloride colorimetric method with quercetin as standard (Chang et al., 2002; Kolasec et al., 2004; Ameer et al., 2010). A solution of 6 mg/ml of *C. serratum* extracts in 80% methanol and different concentrations of quercetin (0.007, 0.015, 0.0313, 0.0625, 0.125, 0.25, 0.5, and 1 mg/ml in 80% methanol) were prepared. Briefly, 500 µl of plant extracts and each concentration of quercetin (Sigma Aldrich, Germany) were pippetted in respective test tubes followed by 0.1 ml of 10% (w/v) aluminum chloride (R & M Chemicals, UK), 0.1 ml of 1 M potassium acetate (Merck, Germany), 1.5 ml of methanol and 2.8 ml of distilled water. The test tubes were thoroughly mixed and after incubating at room temperature (24 to 26°C) for 30 min, the absorba nce of the reaction mixture was measured at 415 nm with a Hitachi U-2000 spectrophotometer (Hitachi, Japan) against blank. The amount of 10% (w/v) aluminum chloride was substituted by the same amount of distilled water in a blank. The concentration of total flavonoid contents of the extracts were determined using a standard curve with quercetin (Sigma–Aldrich Chemie, Steinheim, Germany) (0 to 50 mg/ml) as the standard. The data were presented as mean ± SEM (n = 3).

Determination of antioxidant activity

Free radical scavenging activity

Free radical scavenging activity (FRSA) of *C. serratum* extracts were measured in term of hydrogen donating or radical scavenging ability using the stable 2,2'-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) (Sigma Aldrich, Germany). The method for estimating free radical scavenging activity was adopted from that of Braca et al. (2001) with some modifications (Ameer et al., 2010). Firstly, l00 µl of different extracts of *C. serratum* was pipetted into 96 well plates and serial dilution was made with methanol as blank. Then, 200 µl of methanolic solution of $DPPH^{\bullet}$ (0.2 mM) was mixed with 100 µl of test samples (0.1 mg/ml) into each well plate and incubated at room temperature (24 to 26°C) for 30 min. The absorbance of the mixture was measured at 517 nm against methanol as a blank using microplate reader spectrophotometer (PowerWave X 340, USA). Butylated hydroxytoluene (BHT, 0.01 mg/ml), quercetin (QTN, 0.01 mg/ml), ascorbic acid (water soluble vitamin C, 0.01 mg/ml) and trolox [6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid] (0.01 mg/ml) were used as reference standards. The percentage of radical scavenging activity of the tested samples was evaluated by comparison with a control (100 µl methanol + 200 µl of 0.2 mM $DPPH^{\bullet}$). Each sample was measured in triplicate and the average was taken. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity, and vice versa. The free radical scavenging activity (FRSA) was calculated using the following formula:

$$FRSA = (A_0 – A_1/A_0)*100$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of tested samples after 30 min. The free radical scavenging activity of *C. serratum* extracts, BHT, quercetin, ascorbic acid, and trolox were expressed as EC_{50}. The EC_{50} values are defined as: the amount of antioxidant required by the test samples to cause 50% decrease in initial DPPH• concentration.

Trolox equivalent antioxidant capacity

The total antioxidant activity (TAA) values, were estimated by the trolox equivalent antioxidant capacity (TEAC) method. This assay was estimated by using the 2,2'-azinobis-(3-ethylbenzothiazoneline-6-sulphonic acid) diammonium salt ($ABTS^{\bullet+}$), as a free radical provider for determination of scavenging ability of *C. serratum* extracts according to the method of Re et al. (1999) and Yam et al. (2007). Initially, the preformed radical monocation of $ABTS^{\bullet+}$ solution was generated by reacting (7.4 mM) $ABTS^{\bullet+}$ salt solution in 0.01 M phosphate-buffered saline (PBS), pH 7.4, and oxidizing it, using potassium persulphate ($K_2S_20_8$) 2.45 mM (R & M Chemicals, UK). The mixture was allowed to stand for 16 h in the dark at room temperature (24 to 26°C) before use. The mixture was d iluted to 10 fold with PBS, pH 7.4. Then, absorbance of the reactant was adjusted to 0.70 ± 0.02 at ambient temperature at a wavelength of 734 nm. Stock solution of trolox (0.5 to 4 mM) (Sigma Aldrich, Germany) and *C. serratum* extracts (0.5 mg /ml) were prepared in PBS.

The spectrophotometer (Hitachi U-2000, Japan) was preliminarily blanked with PBS. The plant extracts were dissolved in 80% methanol to yield a concentration of 0.5 mg/ml. The reaction was started, by adding 10 µl of antioxidants containing solution to 2 ml of $ABTS^{\bullet+}$ salt solution. The decrease in absorbance of decolouration (corresponding to a diluted sample) was measured in a dark at 734 nm, 6 min after addition, for trolox or *C. serratum* extracts. All sample determinations were performed in triplicate and the average was taken. The TEAC value is defined as: "the molar concentration of trolox solution having the antioxidant capacity equivalent to the sample solution being tested". This method is applicable for both hydrophilic and lipophilic compounds (Mathew & Abraham, 2006). The capability to scavenge the $ABTS^{\bullet+}$ radical cation was calculated using the following equation:

$$(\%) = (A_1\text{-}A_2/A_1)*100$$

Where A_1 is the absorbance of the control $ABTS^{\bullet+}$ solution without test samples and A_2 is the absorbance in the presence of the test samples.

The antioxidant capacity of the extracts was obtained by comparing the change of absorbance at 734 nm in a test reaction mixture containing extracts with that of trolox. The results reported are mean values expressed as µM of trolox equivalents per mg of test samples.

Antiangiogenic assay (rat aortic model)

The angiogenesis assay was performed according to the manner described by Brown et al. (1996), with slight modification (Sahib et al., 2009). Adult male Sprague-Dawley (SD) rats 12 to 14 weeks of age, and around 160 to 180 g of weight, were used in the experiments. Animals were killed by using carbon dioxide (CO_2) gas and followed by exsanguination. The animals were excised, to isolate the thoracic aorta and placed in PBS. Freshly excised thoracic rat aorta was rinsed with Hanks balanced salt solution containing 2.5 µg /ml amphotricin B (Sigma-Aldrich, Germany). The tissue specimens were then cleaned off adipose tissue materials

and residual blood clots. The rat aortic ring was then cut into small rings of 1 to 2 mm cross-section segments under a dissecting microscope (Motic®, Taiwan). The assay was performed in a 48 well tissue culture plate (Nunclon™, Denmark). A volume of 500 µl of 3 mg/ml fibrinogen (Calbiochem, USA) in serum free M199 growth medium (Gibco®, USA) was added to each well with 5 mg/ml of aprotinin (Sigma-Aldrich, Germany), to prevent fibrinolysis of the vessel fragments. The clean rat aortic ring segments were rinsed five times in M199 growth media and placed in the centre of the each well (1 ring/well) of 48 well plates. Then, 15 µl of thrombin (50 NIH units/ml) (Sigma-Aldrich, Germany) in 0.15 M sodium chloride (NaCl) were added in each well. Bovine serum albumin plasma (Sigma-Aldrich, Germany) was added to the well and mixed rapidly with fibrinogen. Immediately after embedding the vessel fragment in the fibrin gels, 0.5 ml of medium M199 supplemented with 20% heat inactivated fetal calf serum (Gibco®, USA), 0.1% ε-aminocaproic acid (Sigma-Aldrich, Germany), 1% L-glutamine (Sigma-Aldrich, Germany), 1% amphotricin B (Sigma-Aldrich, Germany), 0.6% gentamycin (Sigma-Aldrich, Germany) were added to each well. Suramin, a well-known antiangiogenic agent, was used as a positive control (La Rocca et al., 1990). A solution of equivalent concentration of medium without the sample was added in wells, served as negative control. Vessels were cultured at 37℃ in a humidified CB150 incubator (Binder, Germany) for 5 days. Fresh medium was added on day four of the experiment. The extent of blood vessel growth formation was determined according to the technique developed by Nicosia et al. (1997). Briefly, the length of the tiny blood vessel outgrowths from the primary ex-plant was measured under a microscope using an inverted Olympus LH 50A microscope camera (Olympus, Japan) on day five of the procedure. The pictures of the vessels were captured with the aid of a camera (Lieca CCD, Japan) and software packages (Lieca QWin) connected with an Intel Pentium 4 desktop computer. The percentage of blood vessels growth inhibition was determined according to the following formula:

Blood vessels inhibition (%) = 1 - (Sample growth / Control growth) × 100

Vascular responsiveness

The male SD rats weighing 250 to 300 g were sacrificed by stunning and followed by exsanguination. The chest was opened up by means of a middle incision from neck region down to the abdominal cavity to expose the visceral content and to isolate the descending thoracic aorta. The aorta was rapidly removed, made free from surrounding tissue and placed in a petri dish containing Kreb's physiological salt solutions (NaCl 6.89, KCl 0.37, $NaHCO_3$ 2.1, $MgSO_4.7H_20$ 0.29, KH_2PO_4 0.16, $CaCl_2$ 0.28 and $C_6H_{12}O_6$ 1.1 g/L). After adherent, fatty and connective tissues were cleaned off, and the aorta was cut into 3 to 5 mm long rings. The isolated rat aortic rings were suspended between two hooks in 10 ml double-jacketed organ bath. One hook was connected to the tissue holder while the other hook was connected to a force displacement transducer by a thread for tension measurement. Special caution was taken in other not to stretch the aortic ring excessively or to damage the luminal surface of endothelial lining. The tissue chamber solution was bubbled continuously with a mixture of 95% oxygen and 5% carbon dioxide (carbogen). The isolated rat aortic rings were subjected to an initial resting tension of 1.0 g before experimental protocol was adopted. If needed, the initial tension was re-adjusted to the baseline and then kept constant throughout the period of experiment (Ameer et al., 2009a). The Kreb's solution in the tissue chamber was replaced constantly with fresh Kreb's solution every 15 min intervals to protect against interfering metabolites (Altura and Altura, 1970). All drug solutions were freshly prepared on the day of the experiment. The stock solution of noradrenaline (NA) 10^{-2} M (Sigma-aldrich, USA), containing 20 µg/ml of ascorbic acid (Sigma-aldrich, USA) that prevent oxidation, was prepared. After that, serial dilution of NA (1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7} and 1×10^{-8} M), were prepared from stock solution with Kreb's solution. The test samples were dissolved in 0.5% of polyethylene glycol-400 (PEG-400) (Merck, Germany) and diluted further to desired final concentrations with kreb's solution. The experiment in the absence of test samples was carried out as a negative control. Verapamil (Sigma-Aldrich, Germany) was used as a positive control (Gilani et al., 2005).

The vasorelaxant activity of *C. serratum* extracts were tested against NA-induced contraction on isolated rat aortic ring preparation using the same protocol reported by Ameer et al. (2009a and b). The preparation was allowed to equilibrate for at least 60 min before the start of the experiment. After baseline tension was stabilized, a cumulative dose-response curve of NA was constructed starting with low concentration to a maximum concentration (1×10^{-10} to 3×10^{-5} M). The isolated rat aortic rings preparation was then pre-incubated (20 to 25 min) with a predetermined concentration of test samples in the organ bath. Then, a new cumulative dose-response curve of NA was constructed again, in the presence of the test samples.

Statistical analysis

All data were expressed as mean percentage of maximum contraction ± SEM and obtained from separate (n = 8) experiments. Contractile response in the presence of different concentrations of the test samples was assessed as a percentage of maximum response contraction of NA. The average response values were plotted, to obtain a best-fit dose-response curve with the maximum response of contraction (where, R_{max}, is the effect of maxium agonist-induced response) and pEC_{50} values (negative logarithm of the drug concentration that yield 50% of R_{max}), against logarithmic concentration of the test samples. The R_{max} and pEC_{50} values were calculated with the aid of GraphPad Prism software (GraphPad prism, USA). Statistical analysis was performed using GraphPad Prism software (GraphPad prism version 5.0.1. San Diego, CA, USA).

The significance difference was evaluated with a one-way analysis of variance (ANOVA) followed by Bonferroni/Dunnett post hoc test to compare between groups. In all the cases, values of $P<0.05$ were considered to be statistically significant.

RESULTS

Extraction

In extraction, amongst the four extracts, ME-CS showed highest extraction yield (28.5%). Followed by water (13.6%), chloroform (7.2%), and petroleum ether (4.5%), respectively.

Phytochemical analysis

Preliminary phytochemical analysis of most active extract, ME-CS, showed the presence of significant amounts of polyphenolics (flavonoids, hydrolysable tannins), terpenoids and saponins. In agreement with the study of Banerjee (1969) and Cannon et al. (1980), this study could not detect any alkaloid in ME-CS.

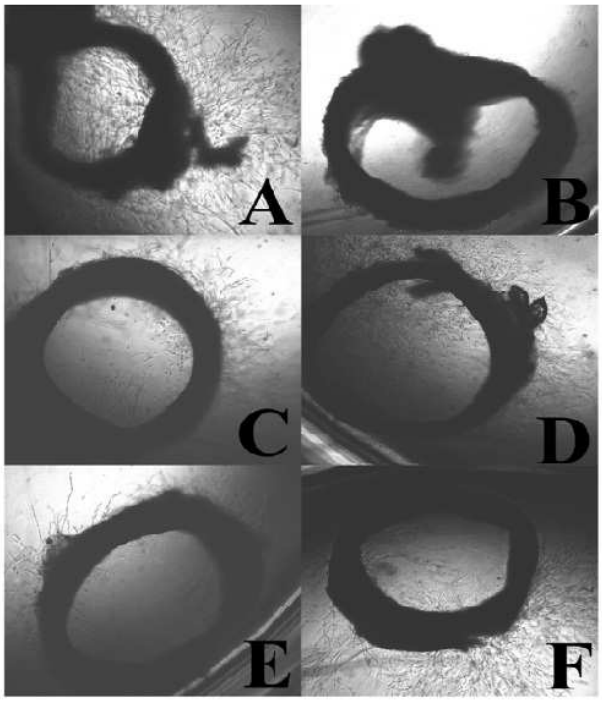

Figure 1. The images of blood vessels growth on rat aortic rings in the medium containing (A) 1% DMSO: dimethyl sulfoxide; (B) suramin; (C) petroleum ether; (D) chloroform; (E) methanol and (F) water extracts of *C. serratum* leaves.

The effect of *C. serratum* leaves extracts on blood vessels growth

The antiangiogenic effect of different extracts of *C. serratum* was evaluated on rat aortic ring preparations (Figure 1). The results obtained on day five of the procedure indicate that, all the extracts of *C. serratum* inhibited blood vessels growth. Amongst the four extracts, ME-CS (45.79 ± 0.16%) shows the most potent antiangiogenic activity significantly (P<0.001), followed by petroleum ether (16.95 ± 0.08%), chloroform (11.86 ± 0.10%) and water (8.47 ± 0.00%), respectively on rat aortic ring assay (Figure 2). Suramin, a well-known antiangiogenic agent, acts as positive control inhibited the blood vessels growth by 98.30 ± 0.08% after five days of procedure (p<0.001).

However, because the ME-CS was most active, it was selected for further study to determine its half maximal inhibitory concentration (IC_{50}) value. The effect of six different concentrations (400, 200, 100, 50, 25 and 12.5 µg/ml) of ME-CS were studied on rat aortic ring assay. These concentrations of ME-CS inhibited blood vessels growth by 100 ± 0.02, 78.0 ± 1.5, 69.02 ± 3.17, 62.03 ± 2.9, 56.01 ± 2.9 and 31.0 ± 5.0%, respectively. The IC_{50} value of ME-CS obtained from logarithmic regression curve equation, [$Y = 17.165\ln(x) - 7.167$ ($R^2 = 0.9453$)] was 27.93 µg/ml.

The effect of *C. Serratum* leaves extracts on NA-induced contraction of isolated rat aortic ring preparations

Polyethylene glycol-400 (PEG-400) is considered an inert substance and possesses low toxicity as a solubilizing agent for poorly water-soluble extracts (Ameer et al., 2009). However, the solubilizing agent should not have any effect on NA-induced contraction of rat aortic ring preparations. This experiment was performed to eliminate the possible effect of PEG-400, as negative control, on isolated rat aortic ring preparation (Figure 3). The PEG-400 at the concentration of about 0.5% did not significantly changed the contractile response in R_{max} value of NA-induced contraction of isolated rat aortic ring preparation (p<0.001). Moreover, it was found that that there were increase in pEC_{50} values (= -logEC_{50}) of PEG-400 on NA-induced contraction on isolated aortic ring preparations (Table 1). Higher values of pEC_{50} values (= -logEC_{50}) indicates exponentially greater potency of PEG-400. Hence, caution should be taken on PEG-400 at the dose more than 0.5% when applied on isolated rat aortic ring preparations. Although, other researchers reported more dose of PEG-400 (Ameer et al., 2009b), on isolated rat aortic ring preparations.

It was found significantly, that drop of contractile responses of R_{max} and pEC_{50} (= -logEC_{50}) values of increasing different concentrations (1.0 and 2.0 µM) of verapamil, was positive control, on NA-induced contractions on rat aortic ring preparations (p<0.05). Moreover, the lower concentration of verapamil (0.5 µM) in tissue chamber did not significantly affect NA-induced contraction of isolated rat aortic ring preparation (Figure 4 and Table 1).

All concentration (0.12, 0.25, 0.5 and 1.0 mg /ml) of petroleum ether extract of *C. serratum* (PE-CS) did not significantly affect NA-induced contraction of isolated rat aortic ring preparations compared to the control (Figure 5 and Table 1).

The lower concentrations (0.12 and 0.25 mg ml^{-1}) of chloroform extract of *C. serratum* (CE-CS) in tissue chamber did not significantly affect NA-induced contraction of isolated rat aortic ring preparation (Figure 6), while higher concentrations (0.5 and 1.0 mg mL^{-1}) of CE-CS in tissue chamber were significantly inhibited NA-induced contraction of isolated rat aortic ring preparations (Table 1). This may indicate the presence of some vasorelaxing elements in CE-CS leaves which may not be active at lower concentrations. In contrast, all concentrations (0.12, 0.25, 0.5 and 1.0 mg/ml) of ME-CS was dependently inhibited and this, shifted the log dose response curve to the right completely with depressed maximum responses, significantly on NA-induced contraction of isolated rat aortic ring preparations (Figure 7). Moreover, significant decrease of contractile responses of R_{max} values and pEC_{50} (= -logEC_{50}) values increase of all concentrations of ME-CS was found (Table 1). This indicated the presence of high concentrations of

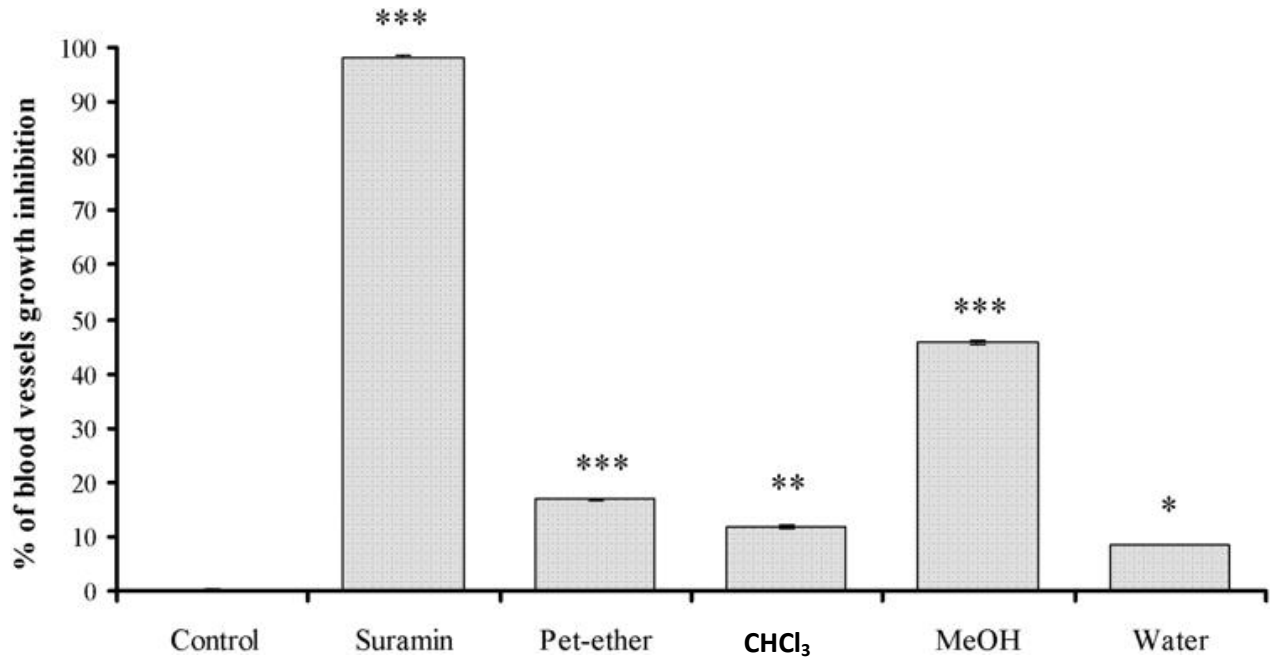

Figure 2. The effect of extracts of *C. serratum* leaves on blood vessels growth inhibition (n=6). DMSO: dimethyl sulfoxide; $CHCl_3$: chloroform; MeOH: methanol. * (P<0.05), ** (P<0.01) and *** (P<0.001).

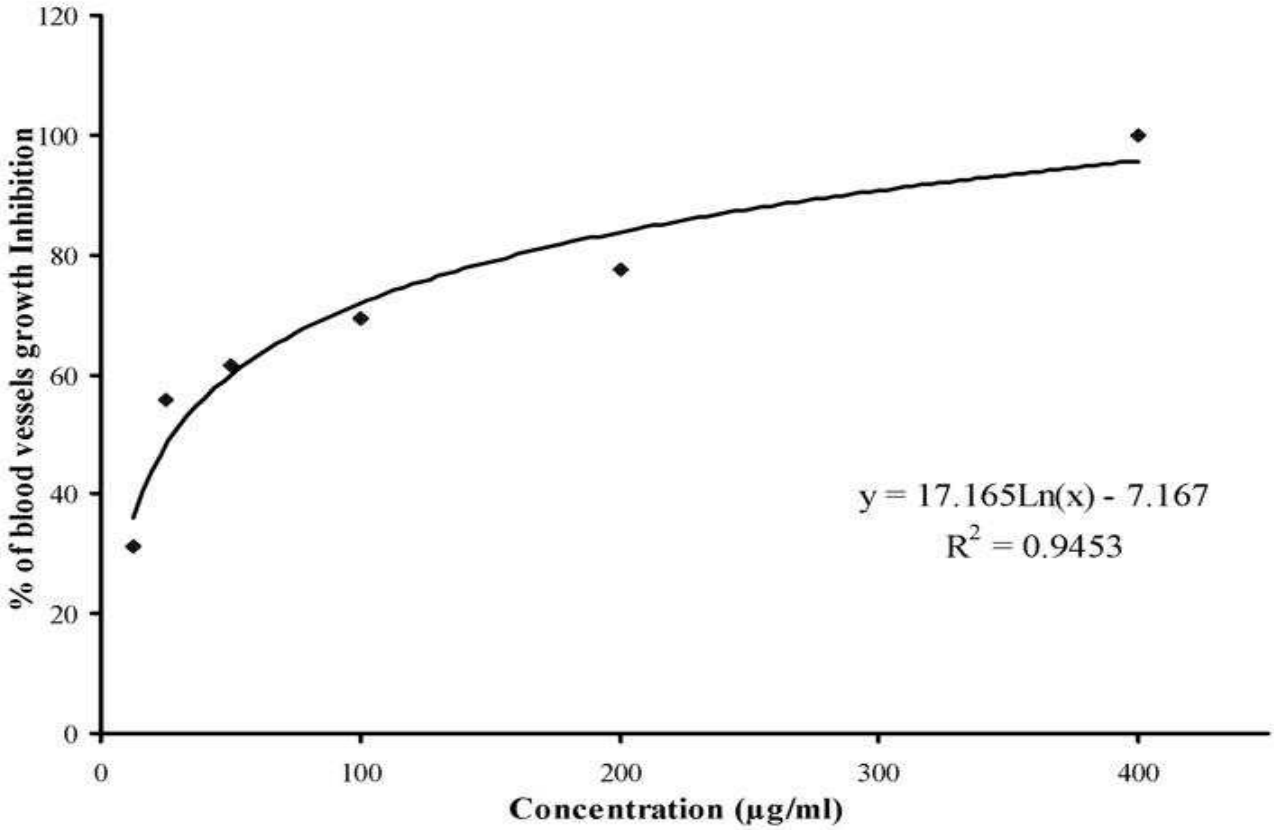

Figure 3. The effect of polyethyline glycol-400 (PEG-400) on NA-induced contraction of isolated rat aortic ring preparations (n=8).

Table 1. The maximum response (R_{max}), and pEC_{50} (= -logEC_{50}) values of NA-induced contractions of Isolated rat aortic ring preparations in the presence of different concentrations of crude extracts from *C. serratum* leaves.

Test samples	R_{max} (%)	pEC_{50} (= -logEC_{50})
PEG-400 (%)		
Without PEG-400	95.66±1.70	7.33±0.05
0.0625	87.85±2.83	7.36±0.10
0.125	88.55±2.10	7.39±0.07
0.25	90.78±2.92	7.46±0.10
0.50	91.05±3.02	7.55±0.10
Verapamil (µM)		
Without Verapamil	98.49±1.29	7.17±0.04
0.5l	55.14±2.20	7.12±0.10
1.0	45.47±2.72*	7.02±0.13
2.0	32.47±2.12*	7.06±0.14
Petroleum ether extract (mg/ml)		
Without Pet ether	97.22±1.23	7.75±0.04
0.125	89.32±1.65	7.48±0.06
0.25	81.73±1.47	7.35±0.05
0.5	85.59±2.49	7.25±0.08
1.0	74.31±2.27	7.07±0.08
Chloroform extract (mg/ml)		
Without chloroform	96.02±0.99	7.91±0.03
0.125	73.15±2.35	7.59±0.10
0.25	65.17±1.84	7.42±0.08
0.5	51.80±1.82*	7.46±0.10
1.0	20.16±0.75***	7.37±0.11
Methanol extract (mg/ml)		
Without methanol	96.25±1.49	7.52±0.05
0.125	51.83±1.75**	7.08±0.09
0.25	28.47±1.62***	7.27±0.16
0.5	2.72±0.64***	7.33±0.68
1.0	0.60±0.26***	9.30±2.12
Water extract (mg/ml)		
Without water	96.75±1.11	7.85±0.04
0.125	92.41±4.89	7.89±0.19
0.25	85.33±2.43	7.50±0.09
0.5	75.87±2.67	7.25±0.10
1.0	37.23±1.83*	6.99±0.13

All data obtained were expressed as mean percentage of maximum contraction ±SEM (n = 8). * ($p<0.05$), ** ($p<0.01$), and *** ($p<0.001$) denotes the significant difference from NA alone (control). EC_{50} was obtained from the concentration-response curve of NA-induced contraction and was taken as the concentration required to elicit 50% drop in the maximum contraction.

promising vasorelaxant agents in ME-CS.The highest concentration (1.0 mg/ml) of water extract of *C. serratum* (WE-CS) significantly inhibited NA-induced contraction of isolated aortic ring preparations (Figure 8) while, the lower concentrations (0.12, 0.25, 0.50 mg/ml) of WE-CS did not significantly affect NA-induced contraction of isolated rat aortic ring preparations (Table 1). In contrast, it was observed, in low dose concentration (0.125 mg/ml)

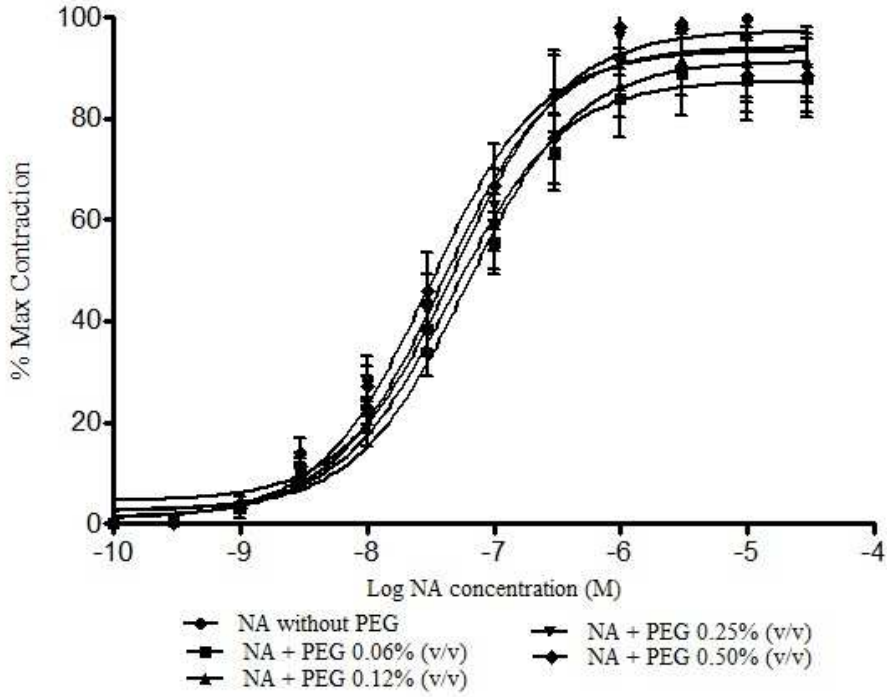

Figure 4. The effect of verapamil on NA-induced contraction of isolated rat aortic ring preparations (n=8).* (*P*<0.05).

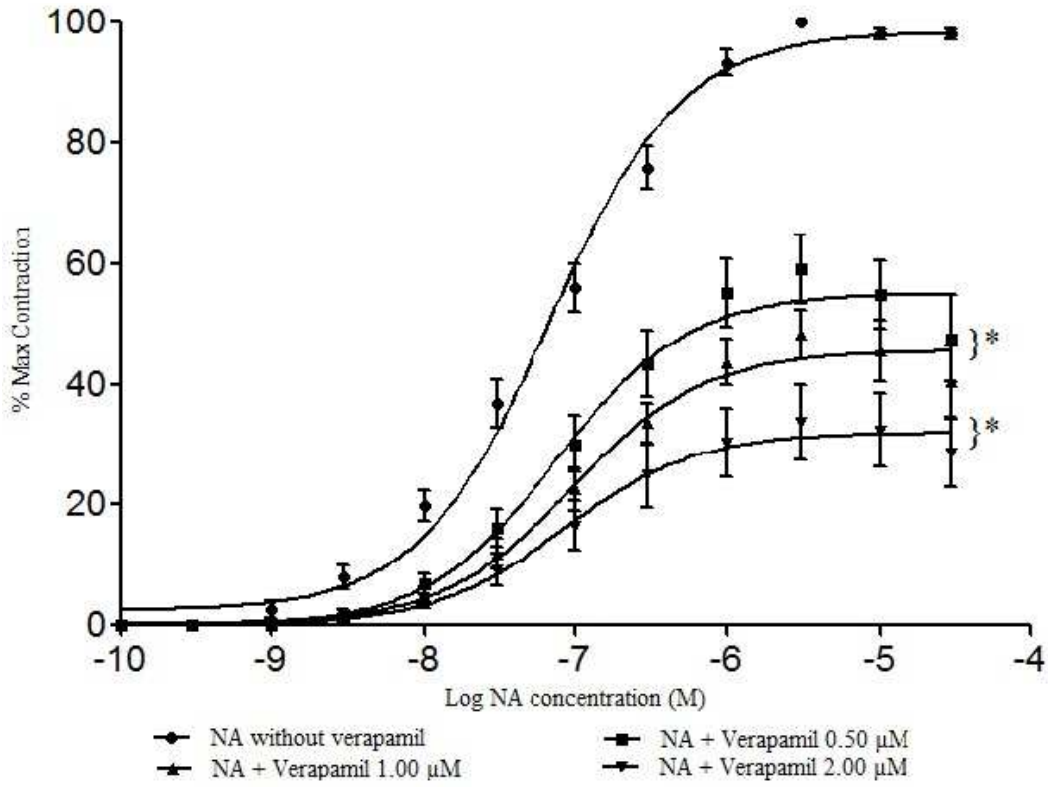

Figure 5. The effect of petroleum ether extract of *C. serratum* (PE-CS) leaves on NA-induced contraction of isolated rat aortic ring preparations.

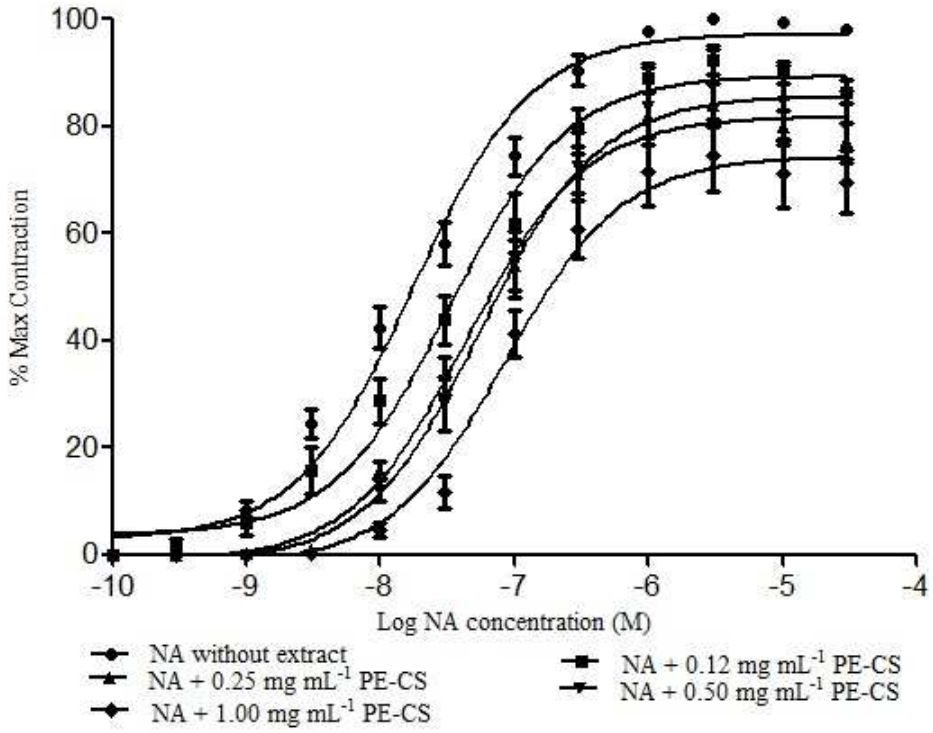

Figure 6. The effect of chloroform extract of *C. serratum* (CE-CS) leaves on NA-induced contraction of isolated rat aortic ring preparations (n=8). * (*P*<0.05), *** (*P*<0.001).

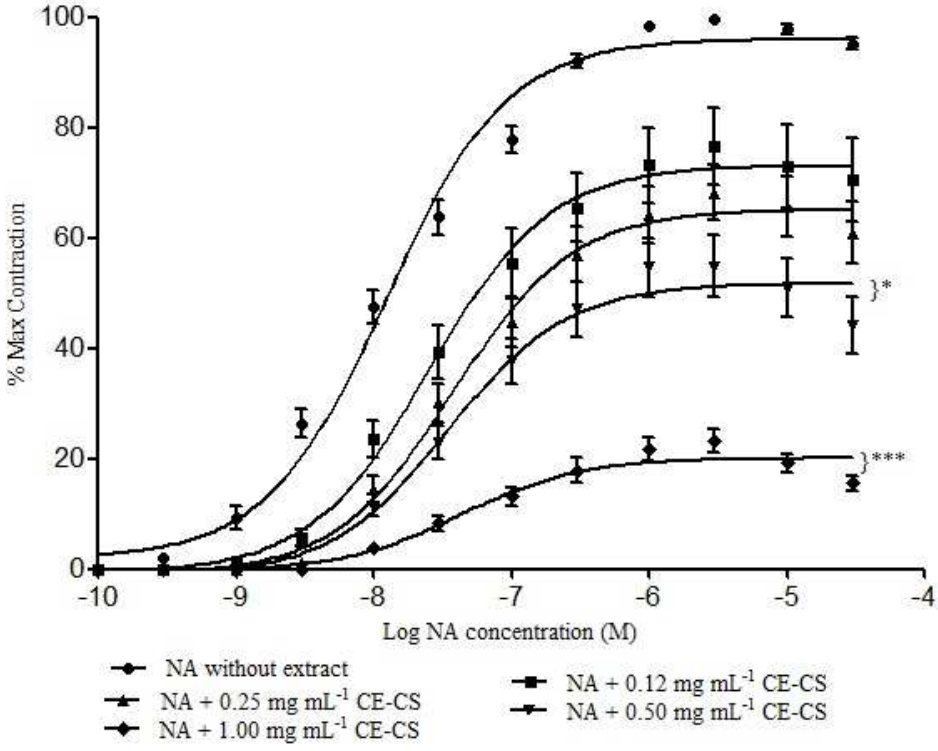

Figure 7. The effect of methanolic extract of *C. serratum* (ME-CS) leaves on NA-induced contraction of isolated rat aortic ring preparations (n=8). ** (*P*<0.01), *** (*P*<0.001).

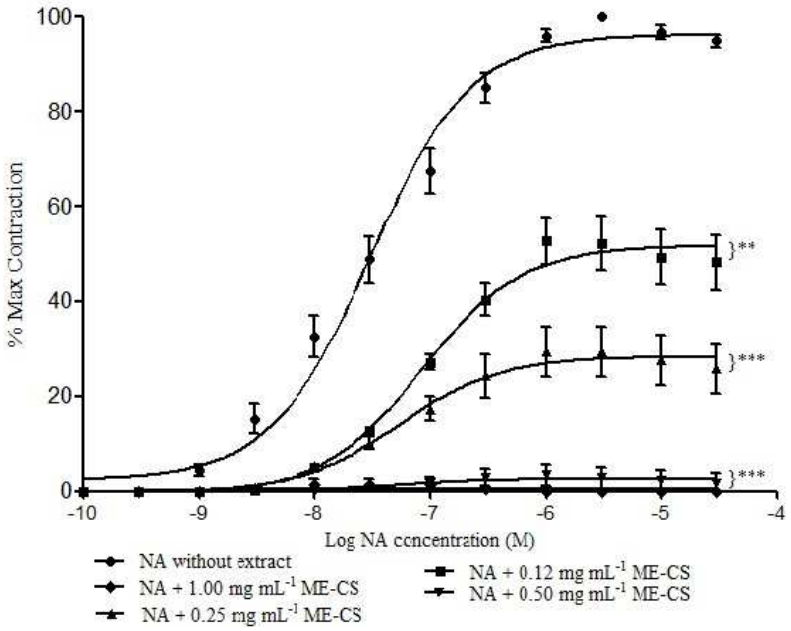

Figure 8. The effect of water extract of *C. serratum* (WE-CS) leaves on NA-induced contraction of isolated rat aortic ring preparations (n=8). * (P<0.05).

Table 2. Polyphenolics contents and antioxidant activity of *C. serratum* (Spreng.) leaves crude extracts.

Crude extract	Polyphenolics		Antioxidant	
	Total phenolics (w/w)	Total flavonoids (w/w)	DPPH$^{\bullet}$ assay	ABTS$^{\bullet+}$ assay
			EC_{50} (µg/ml)	TEAC (mM)/0.5 mg/ml
Petroleum ether	0.0 ± 0.0	0.102 ± 0.20	1.98 ± 0.02	4.83 ± 0.02
Chloroform	3.1 ± 0.4	0.000 ± 0.00	1.03 ± 0.02	5.65 ± 0.10
Methanol	14.6 ± 0.2	0.135 ± 0.00	0.51 ± 0.00	12.90 ± 0.00
Water	11.3 ±1.2	0.321 ± 0.00	0.53 ± 0.00	9.61 ± 0.02
Ascorbic acid	……..	……..	0.013 ± 0.00	.....
Butylated hydroxytoluene	……..	……..	0.258 ± 0.00	.....
Quercetin	……..	……..	0.003 ± 0.00	.....

Each value was expressed as the mean ± SEM (n = 3). EC_{50} values were defined as the amount of antioxidant required by the test samples to cause 50% decrease in initial DPPH• concentration.

of WE-CS, the presence of some agents that augmented NA-induced contraction of isolated Rat aortic ring preparations. Such pattern of dissimilar responses indicated that WE-CS contains contractile enhancing elements as well as vasorelaxant compounds (Ameer et al., 2009a).

Determination of polyphenolics

Total phenolic contents

The amount of phenolics compounds present in *C. serratum* extracts were determined from linear regression equation of calibration curve, [y = 0.834x + 0.0631 (R^2 = 0.9624)] and expressed as gallic acid equivalent in 2 mg/ml of extracts. Amongst the four extracts, the ME-CS was found with the highest amount of phenolic compounds, followed by water extract, respectively. On the other hand, there was no phenolic compound found in PE-CS as lower polar solvent (Table 2). This indicated that the polyphenolics are predominant compounds in ME-CS.

Total flavonoid contents

The total amount of flavonoids contents in *C. serratum*

Table 2. Polyphenolics contents and antioxidant activity of *C. serratum* (Spreng.) leaves extracts.

Crude extract	Polyphenolics		Antioxidant	
	Total phenolics % (w/w)	Total flavonoids % (w/w)	DPPH$^{\bullet}$ assay	ABTS$^{\bullet+}$ assay
			EC_{50} (µg mL^{-1})	TEAC (mM)/0.5 mg mL
Petroleum ether	0.0±0.0	0.135±0.00	1.98±0.02	4.83±0.02
Chloroform	3.1±0.4	0.000±0.00	1.03±0.02	5.65±0.10
Methanol	14.6±0.2	0.102±0.20	0.51±0.00	12.90±0.00
Water	11.3±1.2	0.321±0.00	0.53±0.00	9.61±0.02
Ascorbic acid	……	……	0.013±0.00	.....
BHT	……	……	0.258±0.00	.....
Quercetin	……	……	0.003±0.00	.....

Each value was expressed as the mean ± SEM (n = 3). EC_{50} values were defined as the amount of antioxidant required by the test samples to cause 50% decrease in initial DPPH• concentration.

extracts were determined from linear regression equation of calibration curve, [y = 0.0146x + 0.0351 (R^2 = 0.9989)] and expressed as quercetin equivalents in 6 mg /ml of extracts. Amongst the four extracts, the WE-CS was found with the highest amount of flavonoids contents, followed by ME-CS respectively. On the other hand, there was no flavonoid contents found in chloroform extract (Table 2). This indicated that the polyphenolics are major compounds found in ME-CS.

Determination of antioxidant activity

Free radical scavenging activity (FRSA)

The ability of test samples to scavenge DPPH$^{\bullet}$ was assessed on the basis of the effective concentrations 50% (EC_{50}) values, defined as the concentration of antioxidant (test samples), required to decrease the absorbance at 517 nm of DPPH$^{\bullet}$ solution concentration to half of its initial value. The extracts of *C. serratum* were measured for antioxidant activity, using 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) radicals scavenging assay, and compared with those of the well-known antioxidants such as BHT, ascorbic acid and quercetin. Amongst the four extracts, ME-CS, was found to contain the highest polyphenolic contents, demonstrated highest free radical scavenging activity, followed by water extract (Table 2).

Trolox equivalent antioxidant capacity

The ABTS$^{\bullet+}$ radical scavenging capacity assay was used to examine the antioxidant activity of *C. serratum* extracts. ABTS$^{\bullet+}$ radical scavenging capacities of test samples were expressed as compared with a standard amount of TEAC. Amongst the four extracts, the ME-CS, which contains the highest polyphenolics contents, showed highest activity radical scavenging capacity, followed by the water extract respectively when reacted with the ABTS$^{\bullet+}$ stable radicals. In addition to that, petroleum ether and chloroform extracts showed low radical scavenging activity compared with other extracts (Table 2).

DISCUSSION

The aim of this study was to evaluate antioxidant, antiangiogenic and vasorelaxant activities as well as to study the chemical profiles of *C. serratum* active extract. Our findings, demonstrate that the ME-CS showed the most potent antioxidant, antiangiogenic and vasorelaxant activities as demonstrated by our experiments. Chemical analysis of the most active extract, ME-CS, showed the presence of significant amounts of polyphenolics such as flavonoids and hydrolysable tannins. Therefore, while polyphenolics are the predominant compounds found in ME-CS, it is also highly probable that they may play an important (dominant) role in antioxidant, antiangiogenic and vasorelaxant activity. Since all the three activities of *C. serratum* extracts end up in the same results, it is likely that, all the activities were contributed by the same group (such as polyphenolics) or totally different groups of chemical compounds that may act synergistically together with polyphenolics. The polyphenolic components of higher plants may acts as antioxidant, antiangiogenic, antiproliferative and

antiinflammatory as well as vasorelaxants (Bagchi et al., 2004; Nojiri et al., 2004; Stangl et al., 2007). It is well-established that the antiangiogenic drugs induce vasorelaxant effects in rat aortic ring preparations (Amerini et al., 1997), as found in this study. Apart from the above mentioned vascular benefits, polyphenolics are responsible for the antioxidant, antiangiogenic and vasorelaxant activities of many plants in the green kingdom (Dawidowicz et al., 2006; Erdogan et al., 2007; Mojzis et al., 2008) as herbal therapy.

Polyphenolics are a diverse group of chemical substances that naturally occurs in plants such as flavonoids, tannins and phenolic diterpenes. Plant polyphenolics inhibit angiogenesis through the regulation of multiple signalling pathways (Mojzis et al., 2008) such as angiotensin converting enzyme (ACE) pathway. Hydrolysable tannins'which are ACE inhibitors (ACEI) (Liu et al., 2003; Braga et al., 2007) are proved to be antioxidants (Yamakoshi et al., 1999; Oszmianski et al., 2007). These tannins are known to exhibit good antioxidant and vasorelaxant activity (Mullen et al., 2002). Moreover, ACEI and antioxidants have improved endothelial function in coronary and peripheral vessels (Grover-Páez and Zavalza-Gómez, 2009). Mechanisms whereby the ACEI and antioxidant agents may improve endothelial function include, either reduction of oxidative excess and inflammation (Schiffrin and Touyz, 2003) or by restoring nitric oxide activity (Taddei et al., 1998; Akhlaghi and Bandy, 2009). ACEI possess either indirect or direct antioxidant properties mediated by the stimulation of NO production and simultaneously inhibiting oxidative stress (Münzel et al., 2010). The endothelium-dependent relaxation effect, as found in this study, of polyphenols is mediated mainly by nitric oxide signaling pathway (Akhlaghi and Bandy, 2009). Therefore, antioxidants and antiinflammatory agents that may enhance either NO activity or reduce tissue Angiotensin II is considered therapeutic approach for cardiovascular disease (Dzau, 2001).

The three major enzymatic pathways for arachidonic acid (AA) that stimulate angiogenesis are the cycloxygenase, the lipoxygenase and the cytochrome P_{450} monoxygenase pathways (Pascual et al., 1998; Fleming, 2007). Polyphenolics are inhibitors of AA pathway (Yoon and Baek, 2005). The presence of inflammatory cells, released from AA pathway, is associated with secretion of different angiogenic factors, such as VEGF, basic fibroblast growth factor (bFGF), MMPs, hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), tumor necrosis factor alpha (TNF-α), epidermal growth factor (EGF), angiogenin and transforming growth factor-β (TGF-β), resulting in angiogenesis (Tamarat et al., 2002; Funa and Uramoto, 2003; Chen et al., 2005; Gong et al., 2006). New findings indicated that blood vessels formation and pro-angiogenic factors secretion are inhibited by selective and/or nonselective cycloxygenase 2 inhibitors (COX-2) (Bakhle, 2001), lipoxygenases inhibitors (Nie and Honn, 2002) and cytochrome P_{450} (CYP) epoxygenases inhibitors (Michaelis et al., 2008; Nithipatikom et al., 2010).

Therefore, polyphenolics may be partly responsible for the pharmacological efficacy of several herbal medicines (Tsuda et al., 2004) such as that observed in *C. serratum*. But, only further work will resolve all questions associated with inhibiting blood vessel formation, antioxidant and vasorelaxant activity of *C. serratum* extracts, to fully evaluate the usefulness of these data. Taken together; it can be concluded from this study that, there is strong positive correlation between antioxidant activity, inhibitory activity of methanolic extract to angiogenesis and vasorelaxant activity. To the best of our knowledge, this is the first study that describes the pharmacological basis of the uses of *C. serratum* as antiangiogenic, vasorelaxant properties, as well as antioxidant activity.

In conclusion, these findings suggest that ME-CS could be a promising source of antiangiogenic agent, which induced observed vasorelaxant activity, as well as prevent oxidant potentials of free radicals as a natural source of antioxidants.

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