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Research Article

Free Radical Scavenging Activity of Leaf Extract of *Rumex vesicarius* L. Obtained by Different Methods

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ABSTRACT
In this study, phenolic content, flavonoids and *in vitro* antioxidant activities of leaves extracts of *Rumex vesicarius* L obtained by classical, ultrasonic assisted and Soxhlet method were investigated. The total phenolic content and flavonoids were determined by a spectrophotometric method. Hydroxyl radical, superoxide radical scavenger, DPPH radical-scavenging, Fe^{2+}-chelating activity and hypochlorous acid scavenging methods were applied to test the antioxidant activities. The results indicated that the extract obtained by the Soxhlet extraction showed the highest antioxidant activities and contained higher total contents of phenolic and flavonoids than the extracts obtained by two other extraction techniques (ultrasonic assisted and classical method). From the results that not only the more bioactive components are obtained but also the extract has better free radical and reactive oxygen species scavenging activities through Soxhlet extraction method. These findings further illustrate that Soxhlet extraction has a bright prospect for extracting active ingredients from plant materials. From the above study it is concluded that the ethanolic leaves extract of *Rumex vesicarius* L is a potential source of natural antioxidant.

Keywords: *Rumex vesicarius* L, antioxidant, DPPH, superoxide, hydroxyl, phenolic content

INTRODUCTION

Many diseases like diabetes, liver damage, nephrotoxicity, inflammation, cancer, neurological, cardiovascular disorders and aging are associated with oxidative stress caused by free radicals both reactive oxygen species (ROS) and reactive nitrogen species (RNS) which contains one or more unpaired electrons and can donate or receive electrons to become stable. It can propagate and produce further radicals and also can be made inactive by antioxidants[1,2]. Reactive oxygen species (ROS), such as superoxide radical ($O_2^{\bullet-}$), hydro-gen peroxide (H_2O_2), hydroxyl radical ($HO^{\cdot}$), perhydroxyl radical($HO_2^{\bullet}$) and singlet oxygen ($1O_2$) are the most prevalent free radicals in plants and living cells[3,4]. When the ROS production is excessive, oxidative damage to proteins, lipids and nucleic acids occurs. These alterations are involved in various pathologies and/or complications[5] such as obesity, atherosclerosis, diabetes, cancer, neurodegenerative diseases, liver cirrhosis and the aging process[6]. Antioxidants are usually reducing agents, such as vitamins, carotenoids and polyphenols, which can scavenge reactive oxygen species and inhibit the chain reaction by donating an electron to the free radical. The antioxidant defence system, supported by dietary antioxidants, protects the body from free radicals[7,8]. Plant based antioxidant compounds play a defensive role by preventing the generation of free radicals and hence could be beneficial in alleviating the diseases caused by oxidative stress[9]. In this regard, plants have been a source of medicinal agents for thousands of years, and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine and there is an increasing interest in the therapeutic use of natural products especially those derived from plants[10]. *Rumex vesicarius* L is an annual, glabrous herb of the Polygonaceae, It spreads throughout desert and semi-desert areas of North Africa, Asia and Australia[11], and is distributed widely in the tropics as an ornamental. It is also known as bladder dock, rosy dock, blister sorrel or country sorrel and is mostly cultivated as a leafy vegetable. In South Algeria, *Rumex vesicarius* L is widely used as food, as a medicinal herb[12], it is used in treatment of liver diseases, digestive problems, toothache, nausea, pain, anti-inflammatory, antitumor as well as antischistosomal, and antimicrobial activities[13,14]. It was also found to have aphrodisiac effect[15,16], diseases of the spleen, hiccough, flatulence, asthma, bronchitis, dyspepsia, piles, scabies, leucoderma, toothache and nausea. The plant also used as cooling, laxative, stomachic, tonic, analgesic, appetizer, diuretic, astringent, purgative, antispasmodic and antibacterial agents[17]. The roasted seeds were eaten for cure of dysentery. Finally, the plant can be used also to reduce biliary disorders and control cholesterol levels[18,19]. Despite its importance, only few studies have been conducted on *Rumex vesicarius* L. Thus, the aim of the present work was to

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quantify phenolic content, flavonoids of leaf extracts of *Rumex vesicarius* L obtained by different methods classical, ultrasonic assisted and Soxhlet, and to evaluate the antioxidant activity.

MATERIALS AND METHODS

Plant material

The *Rumex vesicarius* L were collected from southeast of Algeria, state of El Oued on December 2013. The leaves then separated from each other, washed and dried at room temperature. All these organs were ground to a powder with a basic electric grinder and stored in the dark at room temperature before use. Then the powder was put in a hot air oven at 60 °C until complete drying. Depending on the physical characteristics of the samples, the time ranged from 18 at 30 h.

Classical extraction (CE)

Fifty grams of powdered leaves (50 g) and ethanol (300 ml) were put in a series of the Erlenmeyer flasks (500 ml), no additional stirring was applied. The extraction was performed at 30 °C for 60 min. At the end of the extraction cycle the liquid extract was separated from the solid residue by vacuum filtration. The solid residue was washed twice with fresh solvent (60 ml). The filtrates were collected and the solvent was evaporated in a rotary vacuum evaporator at 45 °C. Extracts were stored at +4 °C in dark until use.

Ultrasonic-assisted extraction (UAE)

Ultrasonic apparatus from Branson (40 kHz, 1500W, dimension: 49 cm×14 cm×15 cm) was used for accelerated extraction. A beaker was partially submerged in an isothermal water bath to maintain the extraction temperature at 30 °C. Fifty grams (50 g) leaves were then extracted with ethanol (300 ml) for 60 min. The extract was filtered through Whatman paper on a Büchner funnel by vacuum; the solids were washed with an additional 60 ml of ethanol. The filtrate was rotary-evaporated under vacuum at 40 ◦C to dryness. The crude extracts were dried in a vacuum oven at 45 ◦C. Extracts were stored at +4 °C in dark until use.

Soxhlet extraction (SE)

Fifty grams of powdered were mixed with 300 ml ethanol and extracted in a Soxhlet apparatus for 6 h. The extracts concentrated under vacuum at 40 °C by using a rotary evaporator. To obtain ethanol extracts, air dried powdered plants were boiled with 250 ml of ethanol for 30 min. The ethanol extract were filtered and concentration using rotary-evaporated under vacuum at 45 ◦C to dryness. Extracts were stored at +4 °C in dark until use.

Total phenolic content

The total phenolic contents in all organs were determined by the folin-Ciocalteu method[20]. Briefly, 100 µl of both the sample and the standard (gallic acid) of known concentrations were made up to 2.5 ml with water and mixed with 0.25 mL of 1N Folin-ciocalteu reagent. After 5 min, 2.5 ml of sodium carbonate aqueous solution (2%, w/v) was added to the mixture and was completed the reaction for 30 minutes in darkness at room temperature. The absorbance was read at 765 nm using a UV-visible spectrophotometer (Shimadzu UV-1800, Japan). For the blank the same protocol was used but the extract was replaced by solvent. The concentration of total polyphenols in the extracts was expressed as mg gallic acid equivalent (GAE) per g of dry weight using UV-Visible (Shimadzu UV-1800, Japan) and the equation of calibration curve: $Y= 0.00778x$, $R^2 =0.991$, x was the absorbance and Y was the gallic acid equivalent. All results presented are means (±SEM) and were analyzed in three replications.

Total flavonoids

The determination of flavonoids was performed according to the colorimetric assay[21]. Distilled water (4 ml) was added to 1 ml of leaf extract. Then, 5% sodium nitrite solution (0.3 ml) was added, followed by 10% aluminum chloride solution (0.3 ml). Test tubes were incubated at ambient temperature for 5 min, and then 2 ml of 1 M NaOH were added to the mixture. Immediately, the volume of reaction mixture was made to 10 ml with distilled water. The mixture was thoroughly vortexed and the absorbance of the pink colour developed was determined at 510 nm. A calibration curve was prepared with catechin and the results were expressed as mg catechin equivalents (CE)/g of dry weight.

Hydroxyl radical scavenging capacity[22,23]: A volume a 0.1 ml of EDTA (1 mM) was added to 0.01 ml of $FeCl_3$ and 0.1 ml of H_2O_2. After, the precedent reaction mixed with 0.36 ml of deoxyribose (10 mM) and 1.0 ml of ethanolic extract (various concentrations) or standard ascorbic acid, finally followed by the addition of 0.33 mL of phosphate buffer (50 mM, pH-7.4) and 0.1 ml of ascorbic acid in sequence. After incubation for1 h at 37°C in stand, 1 ml of the incubated mixture was mixed with 1 ml of TCA and 1 ml of 0.5% TBA (in 0.025 M NaOH containing 0.025% BHA) to develop the pink chromogen which was measured at 532 nm, ascorbic acid used for comparison. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation and is calculated using the equation .

Percentage inhibition = [(C-T)/C] x100

Where,

C : Absorbance at 532 nm of the control.

T : Absorbance at 532 nm of the test.

Scavenging activity of superoxide radicals

The superoxide anion scavenging of extracts was estimated using the inhibition of NBT reduction[24] by photochemical generated O_2^-. To the assay mixture contained 2 µM of riboflavin, we added 6 µM EDTA, 50 µM NBT and 3 µg of sodium cyanide in 67 mM phosphate buffer (pH= 7.8) in a final volume of 3 ml. Initial absorbance was measured at 530 nm, the tubes were illuminated uniformly with incandescent lamp at 530 nm. The sample extract was added to the reaction mixture, in which O_2^- radicals are scavenged, thereby inhibition the NBT reduction. Querecetin used as a positive control and the percentage of scavenging inhibition was calculated as: % inhibition = $[(A_{control} - A_{sample})/A_{control}]$ x 100, IC_{50} value is the concentration (µg/ml) of the tested material that causes 50% loss of superoxide radicals calculated by the linear regression analysis.

Figure 1. *Rumex vesicarius* L

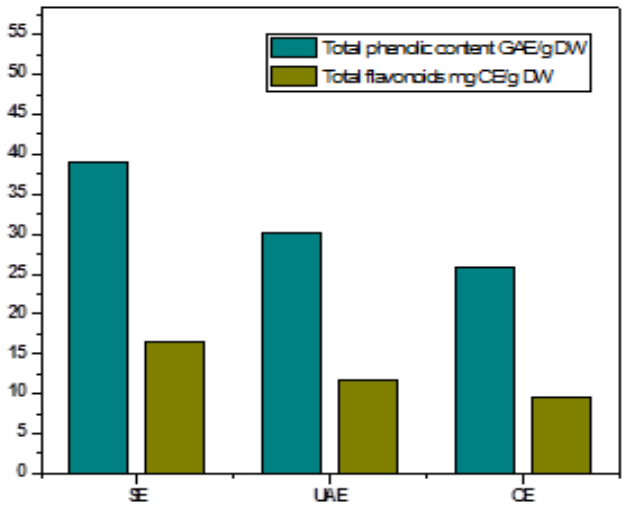

Figure 2. Total phenolic content (mg GAE/g DW) and total flavonoids (mg CE/g DW) of leaves extracts from *Rumex vesicarius* L obtained by different methods.

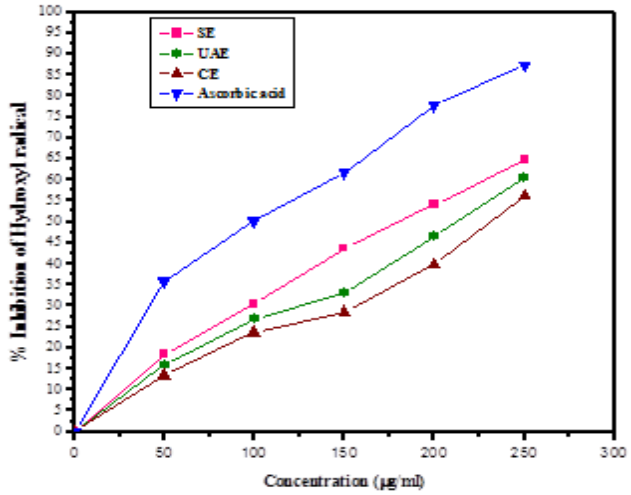

Figure 3. Hydroxyl radical scavenging activity of leaves extracts from *Rumex vesicarius* L obtained by different methods and the standard ascorbic acid

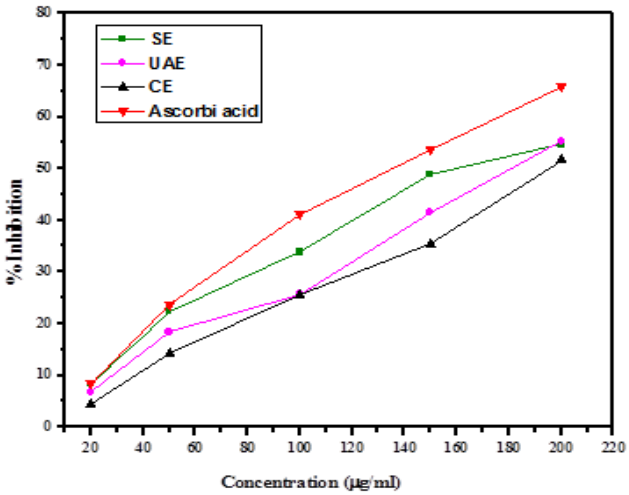

Figure 4. The HOCl scavenging activity of leaves extracts from *Rumex vesicarius* L obtained by SE, UAE, CE and standard ascorbic acid. The data represent the percentage of HOCl inhibition.

DPPH radical scavenging activity

The radical scavenging activity using free-radical DPPH assay was carried out using the spectrophotometric method[25]. 1 ml aliquot of each extract was added to 0.5 ml of a DPPH ethanolic solution (7.8 mg DPPH in 100 mL ethanol 70 %). The mixture was vigorously shaken and left to stand in the dark for 30 min at room temperature. The antioxidant activity was then measured by the decrease in absorption at 517 nm using UV-Visible spectrophotometer and corresponds to the extract ability to reduce the radical DPPH to the yellow-colored Diphenylyldrazine. The Anti-radical activity was expressed as IC_{50} (µg/ml). The Anti-radical percentage inhibition calculated by the following equation:

DPPH Inhibition % =[$(A_o - A_1)/A_o$] x 100, Where A_o is the absorbance of control test after 30 min. A_1 is the absorbance of the sample extract after 30 min. All results are means ±SD.

Metal chelating activity

The chelating activity of leaves extracts from *Rumex vesicarius* L for ferrous ions Fe^{2+} was measured according to the method described by Lucci et al[26]. Briefly, to 0.5 ml of extract at different concentrations, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2 mM) was added. After, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}-ferrozine complex was measured at 562 nm. EDTA was used as positive control. The metal chelation activity was calculated using the following equation: Metal chelating activity (%) =[($A_{control} - A_{sample}$) /$A_{control}$] x 100. where $A_{Control}$ is the absorbance of control devoid of sample and A_{Sample} is the absorbance of sample in the presence of the chelator. The extract concentration providing 50% metal chelating activity was calculated from the graph of Fe^{2+} chelating effects percentage against extract concentration.

Table 1. Scavenging activity of superoxide radicals, metal chelating activity and DPPH of SE, UAE and CE of leaf of *Rumex vesicarius* L and standards (qurerecetin). Antioxidant activity was expressed as % inhibition IC_{50} values (µg/ml) for superoxyde radicals and DPPH

Extraction technique	superoxide radicals IC_{50}= (µg/ml)	DPPH IC_{50}= (µg/ml)	Metal chelating activity (mg EDTA/ g DW)
SE	278.32±8.52	177.58 ±6.85	61.35±1.45
UAE	264.56±8.75	157.28±5.75	45.53±1.54
CE	208.56±7.50	116.54±3.72	39.62±1.14
Querecetin	287.95±8.25	-	

Data are expressed as means ± standard deviation of triplicate samples. Values with different row are significantly ($P < 0.05$).

Hypochlorous acid scavenging

Hypochlorous acid (HOCl) was freshly prepared adjusting the pH of a 10% (v/v) solution of NaOCl to 6.2 with 0.6 M H_2SO_4, and the concentration of HOCl was determined by measuring the absorbance at 235 nm using the molar extinction coefficient of 100 M^{-1} cm^{-1}. The reaction mixture contained, 1.5 mM of HOCl and different concentration of the extract or standard ascorbic acid and incubated for 1 h at 37°C. After that taurine (30 mM) was added and incubate again 30 min at 37°C followed by the addition of thionitro benzoic acid (TNB)[27]. Absorbance was measured at 412 nm against blank and % scavenging was calculated according to the standard formula[28].

Statistical analyses

The data obtained in this study were expressed as the mean of three replicate determinations plus or minus the standard deviation (SD). Statistical comparisons were made with Student's test. *P* values <0.05 were considered to be significant

RESULTS

Total phenolic contents and flavonoids

Ethanolic leaves extracts of *Rumex vesicarius* L obtained by classical, ultrasonic assisted and Soxhlet method were found to be rich in total phenolics and flavonoids contents. The total phenolic content is given in figure 2. SE was found to have the highest value 38.94.15 ± 1.2 mg GAE/g DW, following by the UAE 30.27.45 ± 0.9 mg GAE/g DW and the lowest value in CE 25.76 ± 0.7 mg GAE/g DW.

Similar results were observed in quantification of total flavonoids, the content of total flavonoids was also found to vary significantly ($p < 0.05$) and content ranged from 9.43±0.2 mg CE/g DW to 16.88± 0.5 mg CE/g DW. The Total flavonoids in increasing order was: SE > UAE > CE.

$OH^{\cdot}$ scavenging capacity

Figure 3 shows the percentage of hydroxyl radical scavenging activity of the three extracts obtained CE, UAE and SE. Hydroxyl radical has been implicated as capable of damaging almost every molecule found in living cells[28]. The extracts have shown a dosage-dependent increase in inhibition of $OH^{\cdot}$ radicals. The results show that SE presented the highest antioxidant activity behavior, with values of 181.52±6.5 µg/ml, followed by UAE with results of 212.36±7.82 µg/ml and the lowest value in CE 230.42±8.5 µg/ml.

Superoxide anion radical method

The results for the selected extracts are presented in Table 1 and compared with the standard querecetin value of the superoxyde scavenger capacity. The results for Table 1 are expressed in terms of IC_{50} (mean±standard deviation). The SE show the best results in antioxidant potential, with values of IC_{50}=208.56±7.5 µg/ml, higher than the querecetin standard (IC_{50}= 287.95 ± 8.25 µg/ml). the extract obtained by ultrasonic assisted method shows a moderate antioxidant potential (IC_{50}=264.56±8.75 µg/ml), while the extract obtained by classical method resulted in IC_{50} = 278.32±8.52 µg/ml.

Antioxidant activity by DPPH method

The DPPH method is recommended as a simple and rapid screening method for obtaining basic information about the antioxidant activity of the extracts. It is a commonly and widely used method despite some disadvantages[29]. The SE exhibited the strongest antioxidant activities against DPPH radicals IC_{50}= 116.54± 3.72 µg/ml, followed by UAE IC_{50}= 157.28± 5.75 µg/ml and the lowest value in CE IC_{50}= 177.58± 6.85 µg/ml.

Chelating ability of ferrous ion

The metal ion chelating activity extracts were analyzed and shown in Table 1. The decolorization of red color of the reaction mixture depends upon the reduction of ferrous ions by the plant extracts. The results were expressed as mg EDTA equivalents/ g DW. The metal chelating activity of ethanolic leaf extract by classical method, Soxhlet and ultrasonic assisted extract was 45.5±1.5, 61.35± 1.45 and 39.62±1.14 mg EDTA equivalents/ g DW, respectively. Among the different methods extraction, the Soxhlet ethanolic extract exhibited better metal ion chelating property.

Hypochlorous acid scavenging

Figure 4 shows the dose-dependent hypochlorous acid scavenging activity of CE, UAE and SE of leaf from *Rumex vesicarius* L compared to that of ascorbic acid. The results indicate that the extracts scavenged hypochlorous acid more efficiently (IC_{50} ranges from 157.42 ± 2.56 to 185.34 ± 3.15 µg/ml) than ascorbic acid (IC_{50} = 143.39 ± 1.75 µg/ml) . Similar results were observed for the metal ion chelating activity, the highest inhibition founded in SE IC_{50}=157.42 ± 2.56 µg/ml followed by the UAE IC_{50}=176.91 ± 2.83 µg/ml and the CE IC_{50}=185.34 ± 3.15 µg/ml.

DISCUSSION

In the present study, three extraction methods were used to evaluate the total phenolic contents, total flavonoids and antioxidant activity of leaf extracts from *Rumex vesicarius* L. Among the different solvent extraction methods, successive Soxhlet extraction could provide comparable or even better results than the maceration and ultrasonic assisted for extracting polyphenolic compounds and showed a significant antioxidant activity over the other two methods. Nowadays, several novel extraction techniques such as sonication and microwave-assisted have been developed and employed along with conventional extraction techniques for the extraction of bioactive compounds and nutraceuticals from plants[30]. However, it is important to define clearly which type of capacity is being measured by each one. Moreover, the different actions measured by each method could produce some discrepancies between the results obtained[31]. As a simple assay that provides all the antioxidant information does not exist, it is necessary to apply different methods to obtain the more complete antioxidant profile of the extracts[32]. The preferred extraction method should be simple, fast, economical and able to retain the phytoconstituents of therapeutical and nutraceuticals value. Free radicals are produced in higher amounts in a lot of pathological conditions and involved in the development of the most common chronic degenerative diseases, such as cardiovascul- ar disease and cancer[33], degenerative diseases and also lysis of the cells and tissues. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. Many synthetic drugs protect against oxidative damage but they have adverse side effects. An alternative solution to the problem is to consume natural antioxidants from food supplement as traditional medicines.

Phenolic compounds, tannins and flavonoids have been reported to have multiple biological effects, including antioxidant and anti-inflammatory properties[34]. Recent evidences suggest that diets rich in polyphenolic compounds play a significant role against oxidative stress related disorders because of their antioxidant activities[35]. The hydroxyl radical in the cells can easily cross cell membranes at specific sites, react with most biomolecules and is a highly reactive free radical species and capable of damaging almost every molecule found in living cells[36]. This radical has the capacity to join nucleotides in DNA and cause strand breakage[37], which contributes to carcinogenesis, mutagenesis and cytotoxicity. In addition, this species is considered to be one of the quick initiators of lipid peroxidation process, abstracting hydrogen atoms from unsaturated fatty acids[38]. Superoxide radical is highly toxic species, which is generated by numerous biological and phytochemical reactions. Flavonoids are effective antioxidant mainly because they scavenge superoxide anions[39]. The results suggest that concentration-dependent increasing of superoxide radical scavenging activity. The DPPH free radical is a stable free radical, which has been widely used for estimating the free radical-scavenging activities of plant extracts/antioxidants[36]. The results of the present investigation demonstrate that a successive Soxhlet ethanolic extract can significantly decrease in vitro $DPPH^{\cdot}$ concentration, thus suggesting that plant extract contains secondary metabolites with strong antioxidant activity. Phenolic compounds of the ethanolic leaf extract from *Rumex vesicarius* L are possibly involved in their free radical reactions by reducing the stable DPPH radical to a yellowish colored diphenylpicrylhydrazine derivative.

Transition metal chelating activity depends on the ability of samples to chelate transition metals (Fe^{2+} or Cu^{+}). The ability plays a vital role in antioxidant mechanism since it reduces the concentration of the catalyzing transition metal in lipid peroxidation mechanism. In addition, liposome peroxidation and oxidative damage of protein model systems induced by a Fenton reaction in which ferrous ions catalyze the conversion of hydrogen peroxide to hydroxyl radical with the production of ferric ion. Although metal chelating agents are not antioxidants, they play a vital role in the stabilization of fatty acids against rancidity.

The results obtained revealed that Soxhlet extracts demonstrate a marked capacity for iron binding, suggesting that their action as a peroxidation protector may be related to their iron binding capacity. Because ferrous ions are commonly found in foods, the high ferrous ion chelating ability of the extracts from *Rumex vesicarius* L would be beneficial. HOCl, or its anion hypochlorite (^{-}OCl), is produced in vivo by activated phagocytes in myeloperoxidase–hydrogen peroxide-chloride ion system which is the major strong oxidant generated by the phagocytes cells. HOCl causes fast and important changes in cell shape, electrical resistance, and protein permeability[41]. Moreover, it is capable of deactivating antioxidant enzymes. For example, glutathione peroxidase is very quickly inactivated by low concentration of HOCl. Inactivation of catalase is also rapid, but requires higher HOCl concentrations and the haem appears to be degraded. Hypochlorite is a strong oxidant and reacts quickly with thiols and amines, thus modifying proteins and enzymes[42]. Our results suggest that leaf extract from *Rumex vesicarius* L may ameliorate diseases associated with excessive production of HOCl.

CONCLUSION

In this study, in vitro antioxidant activities, phenolic content and flavonoids of leaf extracts from *Rumex vesicarius* L obtained by classical, Soxhlet and ultrasonic assisted methods have been evaluated. The results indicated that Soxhlet extract exhibited strongest antioxidant activities. The contents of polyphenols and flavonoids Soxhlet extract were significantly higher than those by other extraction methods, which were possibly responsible for higher antioxidant activities of Soxhlet extract. From the results we can draw the conclusion that not only the more bioactive components are obtained but also the extract has better free radical and reactive oxygen species scavenging activities through Soxhlet method. These findings further illustrate that Soxhlet has a bright

prospect for extracting active ingredients from plant materials than the classical and ultrasonic extraction methods.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest related to the publication of this manuscript.

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