Plant Archives Vol. 20, Supplement 1, 2020 pp. 2901-2906 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

ANTIOXIDANT ACTIVITY OF TANNIC ACID PURIFIED FROM SUMAC SEEDS (*RHUS CORIARIA* L.): ITS SCAVENGING EFFECT ON FREE RADICAL AND ACTIVE OXYGEN

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Abstract

The aim of study was to isolate and purify the tannin from the seeds of Sumac (*Rhus coriaria* L.), and then detect the antioxidant characterization. The results of phytochemical analysis of the sumac seeds extract showed the presence of flavonoids, phenols, alkaloids, Terpenoids, glycosides and tannins in the ethanolic extract. Extracted tannin compound by using alcoholic solvents (ethanol/water). followed by using the acetone, were separated tannin by used column chromatograph has been employed by Sephadex LH-20. And tannin was detected by chemical tests: Ferric chloride 1% detector, Lead acetate (CH_3COOPb) 1% detector and purity was confirmed by using High Performance Liquid Chromatography (HPLC). Results of the antioxidant activity of tannin showed that the extract radical scavenging capacity (EC50) values of pure tannin was (9 mg/ml) and possess DPPH radical scavenging activity compared to reference substances BHT (EC50= 4 mg/ml), and this was higher than partial purified tannin component (EC50= 14 mg/ml). which means that the pure tannin of sumac seeds is superior to BHT.

Key words *: Rhus coriaria,* Sumac, Tannin, IC50, Sephadex LH-20, Extracted tannin, High Performance Liquid Chromatography (HPLC), extract radical scavenging capacity (IC50).

Introduction

Rhus coriaria L. (Tanner's Sumac or Sicilian Sumac) is a wild plant growing mainly in the Mediterranean countries, Iran and Iraq. *Rhus coriaria* (Sumac) is a medicinal plant and one of more than 250 species within the Anacardiacea family (Abu-Reidah *et al.,* 2015). Historically, leaves and fruits of *R. coriaria* were known to have remarkable medicinal value in Middle Eastern herbal medicine (Behnammanesh *et al.,*2015). Traditionally, the dark red fruits are dried and ground to produce a crimson sour spice commonly used in Middle Eastern cuisine (Asgarpanah *et al.,* 2014). In addition, extracts of Sumac have been used in the treatment of diabetes, anorexia, haemorrhage and for wound healing (Asgarpanah *et al.,* 2014) . Rapid profiling and isolation of phytochemical compounds within Sumac, reveal its many pharmacological properties. Such active compounds include flavonoids, tannins and xanthons (Regazzoni *et al.,* 2013) all with known anti-microbial, antifungal, hypo-glycaemic and antioxidant and radical scavenging activity (Khalilpour *et al*. 2018). The aim of this study is to isolate and purify the tannin from the seeds of *Rhus coriaria* L, and then the antioxidant effects.

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Materials and Methods

Collection of samples

Sumac seeds were collected from the local market in Baghdad during October, 2018 and classified as *Rhus coriaria* identified by the botanist Assist. Prof. Dr.Sukeyna Abaas Aliwy, Department of Biology /College of Sciences / Baghdad University. Sumac seeds were washed and cleaned from the mud, dust and other plants seeds then placed in the shade inside a well-ventilated room for dryness. The dried seeds were crushed by electric grinder to a fine powder and stored in an airtight container and closed until used.

Preparation of Sumac seeds extract

Preparation of sumac seeds extract was carried out

according to Sheng *et al.,* (2016). About 30 grams of *Rhus coriaria* was shaken with three subsequent mixtures of 95% ethanol and water(1:1 v/v) with the ratio of 1:10 at 80 °C for 2 hours. Insoluble materials which contained very little tannin were removed by filtration with a nylon filter and centrifuged at 3500 rpm for 15 minutes. The ethanol in the solution was removed by a rotary evaporator at 40°C. The remaining tannin was mixed with an equal volume of 1 mM acetate buffer at pH 4, and the remaining aqueous phase was extracted twice with an equal volume of ethyl acetate. The aqueous phase was evaporated to dryness by the rotary evaporator, re-dissolved in a minimum volume of 80:20 ethanol water (v/v).

Identification of Tannin

General tests for tannin acid, standard hydrolysable (tannic acid, Merck) and standard condensed tannins (+)-catechin, Sigma-Aldrich) were assayed for tanning Properties by their abilities to interact with ferric chloride, gelatin, lead acetate and bromine water. Each 2 ml of the solution was mixed with 2-3 drop wises of 1% ferric chloride (Merck), 1 ml of 1% lead acetate (Merck).

Purification of tannin from *Rhus coriaria*

A purified of the residue was performed using open glass column (2.5 X 30 cm) filled with Sephadex LH-20 (Pharmacia Fine Chemicals), The residue was dissolved in absolute ethanol at the flow rate of 0.8 ml/min. and monitored at 280nm. The gel was then washed with 50:50 acetone-water (v/v) at the flow rate of 0.9 ml/min and monitored the absorbance at 540 nm. The 50% acetone fraction, which contained the tannin, was evaporated by the rotary evaporator to remove acetone, and the aqueous solution was extracted three times with an equal volume of liquefied phenol. The aqueous phase was washed with a small amount of diethyl ether to remove phenol, evaporated to dryness, and re-dissolved in a minimum volume of absolute ethanol.

Preparative Thin Layer Chromatography

For analysis of tannin by using Thin Layer Chromatography (TLC) according to Békro *et al.,* (2008), An aliquot of every extract is dissolved in 1 ml of appropriate solvent (generally $CHCl_3$). For TLC, using silica gel sheets (silufol 60 F254, aluminum support; Merck) in appropriate solvent system: $CHCl_3$ /MeOH/ AcOH (18:1:1, v/v/v), revealing - $FeCl_3$ for tannins. Rf was calculated for every constituent.

Thin layer chromatography - Kieselgel GF254 plates, 20 x 20 cm, 1 mm thick, were used. TLC plates were run in duplicate and one set was used as the reference chromatogram. Spots and bands were visualized by UV irradiation (254 and 366 nm) and H_2SO_4 spray reagent.

Analysis of tannin by High performance liquid chromatography (HPLC)

HPLC fingerprints were prepared using a chemito LC6600 model, equipped with isocratic pump and UV-VIS detector. Solvents were pre-filtered by using a millipore system and analysis were performed on reverse phase Lickrospher C18 column (250× 4.6 mm i.d.,5ìm).For injection in HPLC system the active spots were scraped from the reference TLC plates and dissolved in methanol. Injection volume was 5ìl for all the cases. All the extracts were detected at UV wavelength of 318 nm. The flow rate was 1.0 ml /min in all cases.Mobile phase used for different extracts were acetonitrate and acetic acid (70:30) (Lerma-Herrera *et al.,* 2017).

Antioxidant activity of Sumac seed extract

DPPH assay

In the DPPH assay, the antioxidants are able to reduce the stable radical DPPH to non-radical form DPPH-H. (Ajitha *et al.,* 2012).

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The purple colored alcoholic solution of DPPH radical changes to yellow in the presence of a hydrogen donating antioxidant which could be measured at 517 nm. The use of the DPPH assay provides an easy and rapid way to evaluate antioxidants by spectrophotometer (Fereidoonfara *et al.,* 2019). The scavenging reaction between (DPPH) and an antioxidant can be written as: (Kedare and Singh, 2011).

$$(DPPH) + (H\text{-}A) \quad DPPH\text{-}H + (A)$$

(Purple) (Yellow)

The radical scavenging activity of the partial pure and pure tannin was compared at a concentration of (5, 10, 15, 25, 35 and 50 mg/ml). Butylated hydroxytoluene (BHT) and vitamin C were the antioxidants used as positive control. All tests were performed in triplicate and the methanol was used as a blank solution. The percentage DPPH reduction (or DPPH radical scavenging capacity) was calculated as:

% Reduction = (Abs DPPH – Abs Dil.) /Abs DPPH x 100

Where Abs DPPH = average absorption of the DPPH solution, Abs Dil. = average absorption of the three absorption values of each dilution. With the obtained values, a graphic was made using Microsoft Excel.

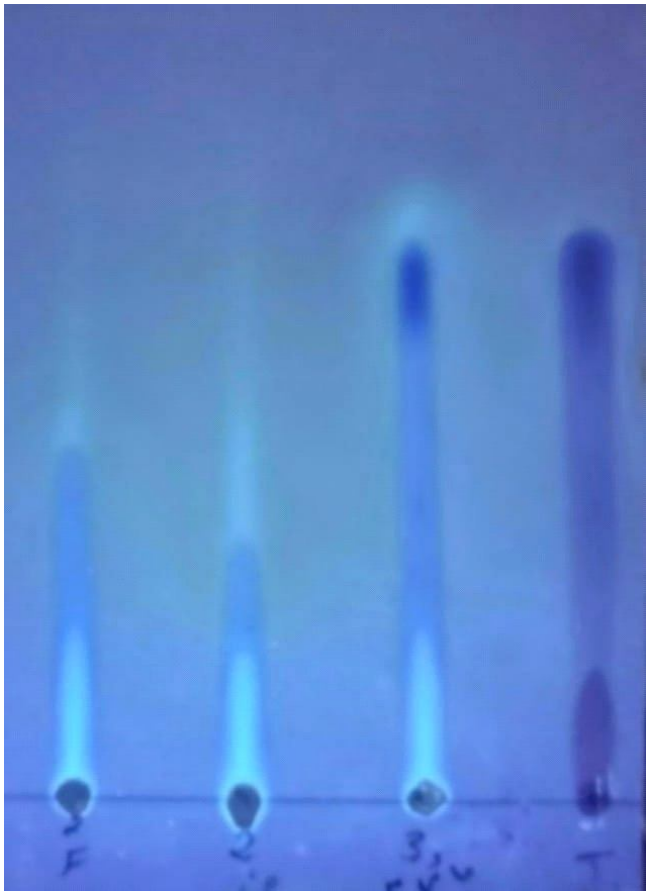
Results and Discussion

This process includes preparation of sumac seed for extraction by removing all foreign materials that they have bad effect on the efficiency and quality of extraction. That may play a false role on this process which will lead to improper result about extraction and purification. Therefore, the delivering from these agents was considered an essential step as a starting point in this project. Extraction starts with increasing sample surface area by grinding seed using electrical mixer to allowing better contact of the extracting solvent with the sample (Anwar *et al.,* 2018), then the ground biomass is ready for extraction.

The solvents which give the best results were high purity 95% ethanol: water (1:1 v/v) at 80°C. (Sheng *et al.,* 2016) patented an alcohol-based method for isolating tannin from sumac seeds in greater than 90% purity. The polymeric powder obtained by ethanol : water extraction of sumac seeds flour is found to release tannin. Temperatures above room temperature (at 80 ºC) are typically used for base hydrolysis. A higher temperature is needed to separate tannin from larger molecular weight compounds such as protein and starch residues which are coagulated and precipitated by the heat.

This step is achieved by using column chromatography Sephadex LH-20 according to Peng *et al.,* (2019) in the purification of tannin from the Sumac seeds. In this study, the purification of ethanolic extract was carried out on this matrix and repeated many times to obtain sufficient amount of tannin. In this technique of purification were depending on molecular weight**.** Sephadex is used to separate low and high molecular weight molecules Sephadex LH-20 is a liquid chromatography media designed for molecular sizing of natural products such as steroids, terpenoids, lipids and low molecular weight peptides (up to 35 amino acid residues) depending on the chosen solvents. Tannins were eluted as a symmetrical peak on Sephadex LH-20**.** There is no single protocol for extracting tannins from all plant material. The procedures used for tannins are widely variable. The acetone solvent in the extraction increases the total yield by inhibiting interactions between tannins and proteins during extraction or even by breaking hydrogen bonds between tannin-protein complexes (Mailoa *et al.,* 2014).

The crud extract of the sumac seeds were subjected to different chemical tests for the detection of different phyto constituents table 1 by using reagents. Thin layer chromatography using Chloroform: water (6:4) as the developing solvent was able to separate different chemicals having different retention factor (Rf value) present in plant extracts. The detection in this method gave the same result characterized by appearing of a dark spot on the silica gel thin layer according to Harbone (1998), where the Rf. value of tannin was 0.923.(Fig. 1). This result was agreement with (Mehta *et al.,* 2017) showed the tannin appear as gray spotlights with Rf (0.92).



(1) crude (2) partial pure tannin (3) pure tannin (4) standard

Fig. 1: Thin layer chromatography plates of *Rhus coriaria* extract the brown zone indicate he presence of tannin.

Tannic acid of Sumac seeds were analyzed by HPLC according to Gupta and Garg (2014) method. The results of the HPLC analysis of *Rhus coriaria* extract showed Tannins with prominent peak with retention time (RT) of 1.937 minutes, (Fig. 2).

One peak have been identified as tannin using standard solution under similar condition (Fig. 3). HPLC analysis revealed the identity of bioactive constituents present in the plant Phytochemical analysis of extracts demonstrated the presence of phytoconstituents like tannin, The medicinal value of the plant lies in bioactive phytochemical action on the human body (Lerma *et al.,* 2017). Some of the most important bioactive phytochemical constituents was tannins, Antibacterial properties of several plant extracts have been attributed to some of these secondary metabolites (Tohma *et al.,* 2019).

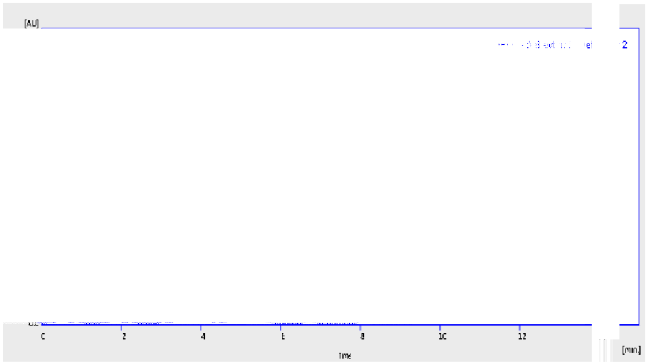


Fig. 2: HPLC chromatography for tannin purification from *Rhus coriaria* .

S. No.	Reten. Time (min)	Area (mAU.S)	Height (mAU)	Area (%)	Height (%)	W05 (min)	Compound Name
1	1.937	45.961	0.704	0.2	0.1	0.03	.....
2	4.960	23451.002	1224.157	99.8	99.9	0.28	Pure Tannin
	Total	23496.964	1224.861	100.0	100.0	.....	

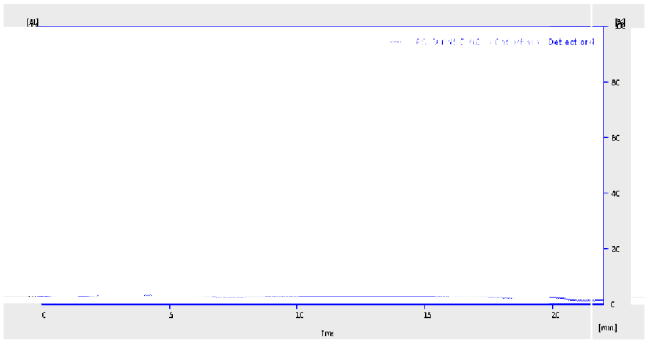


Fig. 3: HPLC chromatography standard.

Antimicrobial action of tannin may be related to their ability to inactivate microbial adhesions, enzymes, cell envelops transport proteins. This potential plant extract (tannin) can be used as therapy for infectious diseases caused by multidrug resistant. (Abu-Reidah *et al.,* 2015).

The pharmacological action of crude drug was determined by the nature of its constituents such as alkaloids, terpenoids, flavonoids, glycosides, saponins and tannins. (Rashid *et al.,* 2016). Finally, those results confirm that RCE is rich in hydrolysable tannins (Farag *et al.,* 2018).

The IC_{50} of each extract (concentration of extract or compound at which 50% of DPPH is reduced) was taken from the graphic. (Al-Jumaili *et al.,* 2019).

The scavenging activity increased gradually with extract concentrations, as for the statistical analysis between different concentrations of the same extract; there was a significant difference at p d" 0.01. The results showed that the pure tannin was the highest free radical scavenging activity from the partial pure tannin with values 4.66 and 4.33 respectively in 50 mg/mL compared to 4.57 for BHT and 4.48 for vitamin C, which means that the pure tannin of sumac seeds is superior to BHT.

Fig. (4) illustrates the concentration of DPPH radical due to the scavenging ability of the extract and standards. The radical scavenging capacity (IC_{50}) of Pure tannin was found to be 9 mg/ml, which is the concentration that decreases the initial DPPH radical concentration by 50%. On the other hand the (IC_{50}) of BHT and vitamin C was 2.5 and 4 mg/ml, respectively, and this was more potent than partial pure tannin component (IC_{50}= 14 mg/ml).

Furthermore, the antioxidant activity is expressed as an effective concentration (IC50). The half maximal effective concentration (IC_{50}) are often refers to the concentration of a drug, toxicant or antibody which induces a response half way between the baseline and maximum after a specified exposure time, it commonly used as a measure of potency of a drug (Chan, 2015).

Lee *et al.,* (2008) reported that if the IC_{50} value of an extract is less than 10 mg/ml it indicates that the extract is an effective antioxidant.

In this study, the IC_{50} value of pure tannin was less than 10 mg /ml, and this indicates that the extract was an effective antioxidant. In addition, it has been reported that the tannins of sumac were to be found as strong antioxidants. The hydrolysable gallotannins are the main tannin compounds present in the Rhus family. The polyol-D-glucose is their basic structural unit, esterified using gallic acid to give the b pentagalloyl-D-glucose, at its hydroxyl groups (Fereidoonfara *et al.,* 2019).

Table 1: Detection of some active compounds in sumac extracts.

Phytochemical compound		Crud Extract	Result
Flavonoids		++	yellow color
Phenols	Ferric chloride	+	bluish green color
	lead acetate	+	reddish brown precipitate
Alkaloids	Wagner's reagent	+	brown precipitate
	Meyer s test	+	White precipitate
Tannins		+++	white gelatin
Terpenoids		+	Brown Color
Glycosides	Benedict's Test	+	Red Precipitate
Saponine		++	thick foam
Steroid		+	

+ = Positive , ++ = good present , +++ = strongly present , – = not detected

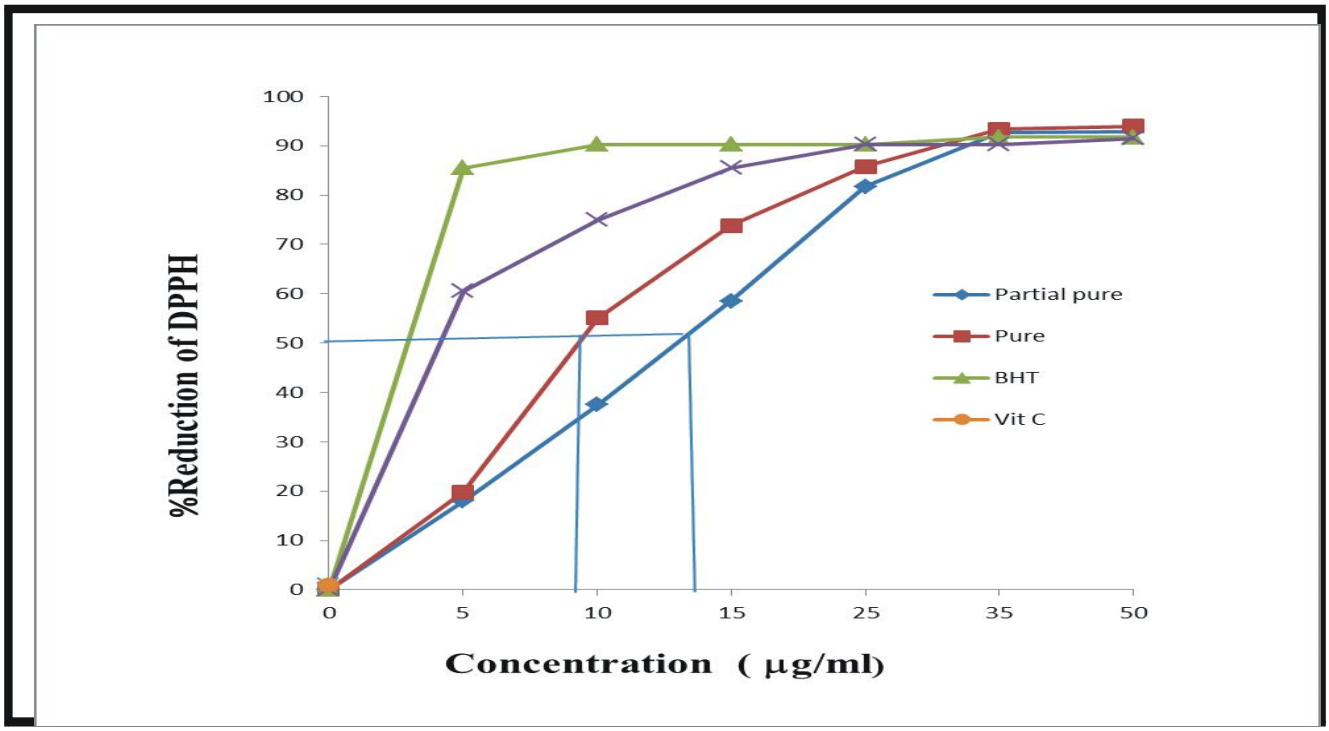


Fig. 4: Percentage of DPPH reduction using Tannin samples (purified and partial purified) and appropriate controls after 30 min of exposure. The corresponding IC_{50} are also outlined.

In conclusion, Sumac seeds available in Iraqi markets can be a good source for tannins, Heating of crude *Rhus coriaria* extract (RCE) at 80^0C /10 minutes before starting purification was considered important step to remove proteins. Tannin possessed high antioxidant effects as free radical scavenger in quenching the DPPH.

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