The Inhibitory Effect of Gallic Acid on Human Serum Cholinesterase

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Abstract

The dried fruit peel of pomegranate in *Punicaceae* family was fractionated chromatographically on Sephadex-LH-20 column .Gallic acid (trihydroxybenzoic acid) and its related galloyl esters such as gallotannin(i.e. β-penta-O-galloyl–D-glucose) were obtained homogenously. Different concentrations of gallic acid and gallotannin were used to determine their inhibitory effect on human serum cholinesterase. The enzyme activity was measured according to the method reported by the WHO .The inhibitory effect of these compounds on the activity of human serum cholinesterase have been studied in vitro .The inhibitory effect was remarkably clear with increasing concentration of gallic acid .Whereas galloyl ester showed no inhibitory effect. The inhibition with gallic acid indicates a noncompetitive pattern. Therefore, we can not recommended gallic acid and its related compounds , as preservative substances in food industry or in pharmacological preparations since they might have some side effect on certain biological systems..

Key words*:* Gallic acid , gallotannin , Human Serum Cholinesterase .

الخلاصة

تم فصل بعض المركبات من القشور الجافة لثمرة الرمان من العائلة الرمانية(*Punicaceae family*) بواسطة العمود السائل اللوني الحاوي على مادة السفادكس ألـ٢٠.و تم الحصول على مادتين متجانستين هما حامض الكاليك (trihydroxybenzoic benzoic acid) وأحد مركبات أسترات الكالوليل التابعة له وهي مادة الكالوتانين(β-penta –O-galloyl –D-glucose) . أن تأثير حامض الكاليك و الكالوتانين لم تدرس بشكل واسع على فعالية ألأنزيمات. استخدمت تراكيز مختلفة من حامض الكاليك والكالوتابين لقياس تأثير تثبيطهما على مصل الكوليستريز(cholinesterase) البشري . أن فعالية هذا الإنزيم تم قياسها طبقا لطريقة منظمة الصحة العالمية (WHO) .. هذه الدراسة اختيرت لتقييم فعالية هذه المركبات مختبريا على مصل الكوليستريز البشري لزيادة معرفتنا حول الفعاليات البيولوجية . أن تأثير تثبيط هذه المركبات جدير بالملاحظة مع زيادة بتراكيز حامض الكاليك بينما لم يلاحظ أي تثبيط معنوي لمادة الكالوتانين . وأن التثبيط بحامض الكاليك تكشف بأنه غير تنافسي بطبيعته .لذلك لا نستطيع أن نوصى بحامض الكاليك والمركبات التابعة له كمواد حافظة في الصناعات الغذائية أو في التحضيرات الصيدلانية نظرا" لاحتمال احتوائها على بعض التأثيرات الجانبية على بعض الأنظمة البيولوجية .

Introduction

Phenolic compounds are secondary plant products which rarely occur in the free state in living plant tissue. Simple phenols are caustic substances and well known to be anti-microbial agents[(1-3)]. Polyphenols like lignin and tannins are also found in plant cells. Tannins or tannic acids are believed to be the most important group of secondary metabolites involved in plant defense[(4-6)] . It has been found that tannins have shown potential antiviral[(7,8)], antibacterial [(9,10)] and antiparasitic effects [(11)]. In the past few years tannins have also been studied for their potential effects against cancer through different mechanisms [(12-14)] . Tannic acids are not single homogeneous compounds, but a mixture of esters of gallic acids with glucose whose exact composition varies according to their sources[(15)] . The biological activation of gallic acid and its related galloyl esters have not yet been studied widely regarding their effect on enzymes. They are employed in medicine as astringents in the gastrointestinal tract (GIT) and on skin abrasions. In the treatment of burns, the proteins of the exposed tissues are precipitated to form a mildly antiseptic, protective coat under which the regeneration of new tissues may takes place[(16)]. Many plant species native to Iraq are known to contain certain chemical compounds which exert their effects on different biological system within the cellular level such as enzymes[(17-19)]. Chemically, these complex substances are usually occur as a mixture of polyphenols that are difficult to separate because they do not crystallize , the application of some chromatographic methods has enabled to confirm the complicated nature of these polyphenolic extracts and also to identify the simple phenols present in small amounts in such mixtures[(20,21)]. In addition, it is of interest to improve methods of separation and identification of gallic acid and some of its related esters obtained from Iraqi plants . Aim of this work was conducted to study the effect of gallic acid and its related glucose esters such as gallotannins on human serum cholinesterase in vitro.

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Materials and Methods

Extraction of plant material:

The fruit peel of Punicaceae in pomegranate family were obtained from Iraqi market. The peels of healthy fruit were dried at room temperature for at least six months, before they were powdered in a mortar and the powder was sieved through 100-150 mesh sieve.The powder (50g) was boiled for few minutes in 100ml ethanol (95%) and left with stirring for at least 3hr. The extract was decanted through fiber glass. The remaining residue was re-extracted twice with ethanol and the combined extracts were concentrated in vacuo to remove ethanol, the heavy viscous residue obtained as the phenolics materials[20,21].

Isolation and identification of phenolic substances:

Phenolic materials were chromatographed on Whatman No.1 paper in two dimensions with 6% (V/V) acetic acid (Solvent A) and isobutanol-acetic acid-water (14:1:5) (Solvent B)at 25±1°C . Phenolic compounds such as gallic acid and gallotannin (Tabel-1)were revealed by spraying with a freshly prepared reagents[15,21-23,] of ferric chloride-potassium Ferricyanide ,Gibbs reagent ,Saturated aqueous potassium iodate(KIO_3) reagent , and finally a fresh solution of nitrous acid reagent was prepared to give the phenolic compounds a characteristic color which helps in their identification . The phenolic extracts (10g) obtained as mentioned above was fractionated chromatographically on Sephadex LH-20 column (100x2.5cm) using the same methods as described previously[21,24]. Sephadex LH-20 is very useful for separation tannin from nontannic phenols[25] .Table-2 showed two substances (i.e. gallic acid and gallotannin) were obtained homogenously by fractionation .

The resultant substances ,as in the following :

1-Gallic acid : Fractions 2 (130 ml) was dried at 25°C and 0.01mmHg over phosphorous pentoxide and rechromatographed once again over Sephadex-LH-20 column . The dried substance gave a pale-yellow-white form melting point (m.p.) 250-253°C. Rf values, showed 0.52 and 0.6 with solvent A and B respectively .Table-1 shows paper chromatograms when treated with a freshly prepared reagents revealed a characteristic colors exhibited by this compound. So under short -u.v. light (254nm) the chromatogram. gave soft blue- violet appearance in visible light which turned to deep violet upon exposure to fuming ammonia. Elemental analysis found ; C, 44.73% ,H, 4.34 %, calc. for $C_7H_6O_5$, as amorphous compound ,(Lit[25] C , 44.70 % ,H,4.31%).

2- Gallotannin;.:Fraction 6 (105 ml) was dried and then rechromatographed in the same procedure as did for gallic acid . The dried substance gave an off-white granular solid; m.p. 200-210°C. Rf values on paper chromatogram showed 0.06 and 0.5 with solvents A and B, respectively.Table-1 shows a characteristic colors exhibited by glallotannin on paper chromatograms when sprayed with reagents .Gallotannin normally showed up as brown-purple spot on chromatograms when treated with Gibbs reagent. The chromatogram also showed a pink appearance with ferric chloride-potassium ferricyanide in visible light which turned to dark- blue absorption in u.v. light which enhanced by fuming with ammonia .Specific spray for gallotannins is Potassium iodate solution, which gives a rose –pink color and reacts with gallic acids to form the characteristic orange of purpurogallin carboxylic acids[26,27]..Elemental analysis showed; C, 52.38 % ; H , 3.48 %; calc for $C_{41}H_{32}O_{26}$, as amorphous compound , (Lit[25].C,52.35 %,H, 3.50 %) .

Table(1) : Detection of gallic acid and gallotannin by various sprays .

Compound	Spray				u.v light
	Ferric Chloride-Potassium ferricyanide	Gibbs reagent	potassium iodate	nitrous acid	
Gallic acid	Blue	Brown	Orange→red→pink	Brown	Blue-violet→deep ep violet
gallotannin	Pink	Brown-purpule	Rose →pink→orange	Brown	Dark blue

Enzyme activity determination:

Different concentrations (ranging from 6mM/L to 30mM/L)of gallic acid and gallotannin were used to determine their inhibitory effect on human serum cholinesterase in vitro. The enzyme activity was measured according to the method reported by the WHO[28], with minor modification as described in previous study [17]. Enzyme activity was expressed as µ moles of substrate (acetylthiocholine iodide) hydrolyzed per ml of total mixture per min.

Results and Discussion

Chromatographic results for the fractions of the extract to detect the phenolic substances as compared with authentic compounds (Tables-1 and- 2) showed that fraction 2 was identified as gallic acid, whereas fraction 6 identified as gallotannin .Also the u.v. light detection ,various sprays, melting points and elemental analysis were applied to identify these compounds . These findings agree with standard authentic compounds were obtained from pharmacy college stores (i.e. gallic acids and gallotannin) which showed in such close agreement as to indicate the identity of the substance previously .Also these finding agree with earlier reports [15,20 ,25,27] No clear inhibitory effect could be detected with different concentrations of gallotannin (ranging from 6 mM/ L to 35mM/l) on human serum cholinesterase. This might be attributed to the presence of amber color which may interfere with the color developed as a result of enzymatic reaction. Decolorization of this solution might merit different results.The inhibitory effects of different concentrations of gallic acid on the enzyme activity were summarized in Table ٣ .It was found that increasing gallic acid concentrations will accordingly affect enzyme activity. Gallic acid concentration as low as 6mM/L results in approximately 10% inhibition ($p<0.001$) and reaching to about 50% inhibition with increasing concentration as high as 30 mM/L ($p<0.0001$). Such highly significant inhibition was unexpected firstly, because gallic acid which was considered as one of the phenolic compounds used as antimicrobial agent in industry[20,29], and secondly, it has not been reported before that phenolic compounds derived from native Iraqi plants exert such inhibitory effect on this enzymatic system i.e. human serum cholinesterase , a well-known biological function of being a neurotransmitter in animals and insects[30] , its has a very high catalytic activity , that catalyzes the hydrolysis of the neurotransmitter acetylcholine into choline and acetic acid, a reaction necessary to allow a cholinergic neuron to return to its resting state after activation[31] .Furthermore, the reciprocal Line weaver-Burk plot for the rate of reaction versus substrate concentration in the presence and absence of gallic acid (Fig.1) showed that the inhibition follows a noncompetitive pattern. This might be explained by that the inhibitor can not bound to the anionic site in the catalytic centre of the enzyme[32]. The mechanism of action of such bounding might be explained through the hydrogen bounding between the carboxyl group of the inhibitor and some catalytically significant group of the enzyme probably with the imidazole moiety of histidine in the static site of the enzyme molecule[33] .

Table(2) : Fractionations of gallic acid and gallotannin by column chromatography using Sephadex LH-20

Fractions	Volume (ml)	Elution Solvent	Weight after drying (g)	Substance
1	٢١٢	Ethanol (100%)	5.56	Unknown
2	١٣٠	Ethanol (100%)	1.42	Gallic acid
3	١٣٧	Ethanol (90%)	1.70	Mixture of gallic acid and other phenolic substances .
4	١٢٥	Ethanol (90%)	-	
5	١١٨	Ethanol (90%)	-	
6	١٠٥	Ethanol (70%)	0.68	gallotannin
7	١٩٥	Ethanol (60%)	0.54	Polyphenolic substances

Table(3) :In vitro inhibition of human serum cholinesterase by different concentrations of Gallic acid.

Inhibitor mM/L	Enzyme Activity U/ml	% Inhibition	Recovery %
Nil	5.93 +1.8	Nil	100.00
06	5.44+ 1.6	08.22	91.69
12	4.76+ 1.4	91.69	80.36
18	4.32+1.3	27.24	72.74
24	3.83+ 1.3	35.67	64.52
30	3.24+ 1.1	45.15	54.38

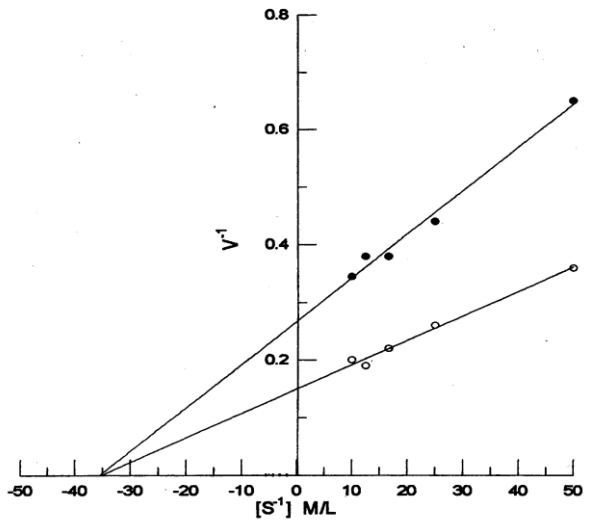

Figure (1) : Double – reciprocal plots for the inhibition of human serum cholinesterase in the presence (●) and absence of (o) of 30 mM/L gallic acid . Acetylthiocholin iodide concentrations (s) were ; 0.02, 0.04 ,0.06 ,0.08 , 0.10 M/L .

Conclusion

Phenolic compounds, such as gallic acid derived from native Iraqi plants with antimicrobial activity might not be recommended to be used as preservative in food industry or in pharmacological preparations since they might exert some undesirable effects on certain enzymatic system such as serum cholinesterase.

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