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SEPARATION OF POLYPHENOL OXIDASE (PPO) AND IDENTIFICATION OF SOME ACTIVE BIOLOGICAL COMPOUNDS IN PROSOPIS FARCTA LEAVES

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ABSTRACT

Prosopis farcta has been used for decades in the folk medicine in north Iraq against a wide range of diseases including, diabetes, diarrhea, hyperlipidemea and intestinal colic. For this reason, many phenolic compounds have been separated and identified.

The (PPO) enzyme, which has a vital role in the formation of polyphenols has been also separated and characterized using different chromatographic methods.

The biological active compounds such as myricetin, quercetin, kaempferol have been detected and separated using different methods. Most of these compounds are potent antioxidants that provide health benefits beyond their ability to neutralize free radicals.

The compound beta-sitosterol, which possesses an anti-inflammatory effect and has ability to lower both LDL and total cholesterol level in blood circulation, has been also separated and identified using different spectroscopic methods.

INTRODUCTION

Prosopis farcta is used in the folk medicine in north Iraq against a wide range of diseases **[Husni (1983)]**. Not only the leaves but also the fruits and the seeds of fruit are used .The fruits are used for neutralization of the acidity existent in the gastric stomach **[Townest *et al.*, (1974)]**.

The decoction of seeds is used as a bandage in order to dissolve the tumor. Because the polyphenols such as flavonoids have important effects on various biological systems **[Hollman (1997)]**; the enzyme polyphenol oxidase [E.C 1.14.18.1] which catalyzes the oxidation of

mono and diphenol compounds to quinones has been isolated and purified by different chromatographic methods such ion-exchange chromatography and gel filtration. The enzyme has been also characterized to find its optimum pH, substrate specialty, optimum temperature and effect of different ion on its activity. Compounds such β-sitosterol has been separated from the leaves, which has many biological importance **[Senatore *et al*, (1989) and Finar (1975)]**. Flavonoids such as myricetin, quecetin and kaempferol which have a variety of biological effect have been separated by chromatographic methods and identified by spectroscopic methods **[Hollman *et al*, (1997) and Agullo (1997)].**

Materials and methods:

The leaves of *Prosopis farcta* have been collected in July 1999 in City Erbil .The leaves were dried and stored at $4C^0$ and grounded by a Wiley mill.

Extraction of ploy phenol oxidase:

Many methods have been used for enzyme extraction but the best one was the acetone powder method according to the method of Prabha *et al*, **[Prabha *et al*, (1984)]** as follow: 40gm of leaves were homogenized in a Warring blender with 400ml-chilled acetone. The suspension was immediately filtered through a buchner funnel. The powder was washed further with 200ml of acetone, air dried and stored at 4°C.

To 20gm of the powder, 150ml of an extraction medium formed from phosphate buffer (0.2M, pH 7.2), containing 1% Triton X-100, 1%(PEG 6000) and 0.01% ascorbic acid were added and stirred for 3min. The extract was filtered through cheesecloth and centrifuged at 4000xg for 30min. in a Beckman refrigerated centrifuge.

The polyphenol oxidase was extracted by the modified method of Jayaraman **(Jayaraman *et al*, (1987)]** by homogenizing 25gm of the powder in 0.2M phosphate buffer, pH 7.2 containing 1% anionic detergent (SDS) at 4°C ,for 3min.The extract was filtered through cheesecloth and centrifuged at 4000 xg for 30min at 4°C, using a Beckman refrigerated centrifuge.

To 60 ml of the clear supernatant 300 mg of calcium acetate was added to remove the polyphenol compounds that were left in the powder after acetone treatment. After stirring for 15 min the clear supernatant on

centrifugation was collected and the precipitate discarded. The supernatant solution was dialyzed against 0.005 M phosphate buffer (pH 7.2) for 3 hrs to remove the calcium acetate, then freeze-dried and chromatographed on sephadex G-75 which was also equilibrated in the same buffer. Fractions were collected using LKB Ultro-Rac7000 and LKB 8300 Uvicord II fraction collector provided with a chart recorder.

The two active peak fractions, which were resulted from gel filtration designated as S-II and S-III, were pooled and separatly chromatographed on DEAE-Sepharose CL 6B column (2.5 x 24cm), which have been equilibrated in the same buffer. The columns were washed with linear gradient of phosphates (200ml of 0.05M phosphate, pH 7.2) in the mixing chamber and 200ml of 0.4M phosphate, pH 7.2 in the reservoir.

Protein determination:

The protein quantity of the enzyme was determined by the method of Lowry **[Lowry *et al*, (1951)]** using bovine serum albumin as standard.

Enzyme assay

The method of Mayer **[Mayer *et al*, (1966)]** was modified for this purpose. The reaction mixture contained 1ml of 0.1M phosphate buffer pH 7, 0.5ml distilled water, 0.5ml enzyme (protein) ranged from 10µg to 2250µg depending on the fraction used, was incubated for 5 min. at 25C° followed by addition of 1ml of substrate. The enzyme activity was measured spectrophotometrically at 395 nm against blank prepared under same conditions. Specific activity was found to be equal to 0.01 OD at 395nm per min per mg protein.

optimum PH:

The buffers used were, citrate pH 3.5 – 6, phosphate $NaNO_2$ pH 6- 7.5, glycine/ NaOH pH 8-9.5 each at 0.05 M concentration. The optimum pH were determined for the enzyme samples collected from peaks DE-I, DE-II which were resulted from DEAE- Sepharose CL-6B respectively.

Optimum temperature:

PPO activity was measured in phosphate buffer (pH 7.0) at a 20C° to 60C° , using catechol as a substrate .

Substrate specificity:

Method of Venkaih and Palwardham **[Venkaiah *et al*, (1977)]** was followed to determine the substrate specificity, using 3.3mM of each of the following substrates: pyrogallol, m-cresol and catechol.

Effect of divalent cation:

The method of Habaguchi **[Habaguchi (1979)]** has been used .0.2mM of the following salts was used to determine the effect of $Ca(NO_3)_2$, $MgSO_4$, $ZnSO_4$, $MnCl_2$, $CuSO_4$, $NiCl_2$ and $FeCl_2$ on the enzyme activity.

Extraction of pectin:

Pectin was separated from the leaves of *P.farcta* and purified **[Harborn (1984)] .**

Extraction, detection and identification of β-sitosterol:

The extraction of β-sitosterol was achieved using different methods. **[Harborn *et al*, (1969)]**. R_f values and quantitative determination of β-sitosterol have been done **[Karting *et al*, (1970) and Ikan (1965)]**; the UV, infrared and ^1H NMR also were used for β-sitosterol identification.

Extraction, detection and identification of flavonoids:

The method of Goodwine **[Goodwine (1976)]** has been used for extraction of flavonoids , then the separation of flavonoids was performed using two- dimential paper chromatography by using (whatman No.1), the chromatogram was run in both alcohol-acid mixture BAW(4:1:5) and in forstal solvent. The markers used were quercetin, kaempferol, and myricetin. The chromatogram was examined for flavonoids by R_f values under visible long wave UV lights, infrared spectra and NMR spectra.

Lipid lowering effect of β-sitosterol:

The unsaponifiable fraction of *P.farcta* leaves which contains β-sitosterol has been tested to show its influnce on the level of some blood

parameters such as cholesterol, low density lipoprotein (LDL) , high density lipoprotein (HDL) and triglyceride (TG). The parameters were analyzed by an enzymatic CHOD-PAP method using test kit of bomeriux-sa (69280 marcel /france).

Animal treatment:

Male domestic rabbits were used in an experiment in April. 2000. The rabbits were adapted for five days before the start of the experiment. During the adaptation period and the experiment, the rabbits were housed in the animal house of the college of science at room temperature 23±3 C°. The animals were divided into two groups with approximately the same weight distribution (1.350-1.550) Kg in each group.

Group I: Control group six Rabbits, non-treated with β-sitosterol fraction.

Group II: Tested group six Rabbits received 100mg/10ml of β-sitosterol fraction per animal/ day.

Water suspension of the dried of β-sitosterol fraction was given orally using special stomach tube, at 8.30 a.m and 8.30 p.m every day for a week.

RESULTS AND DISCUSSION

Results of separation of PPO activity by Sephadex G-75 gave three peaks (S-I, S-II and S-III) .The PPO activities were absent in peak (S-I) , while the activity was present in other two peaks (S-II and S-III) , comprising (39 and 35%) of the total enzyme activity respectively . The purification factors in this stage were (3.27) and (4.45) – fold for both SII and SIII respectively (Table 1).

The ion exchanger separated the S-II peak obtained from gel filtration to three peaks with distinct activity in one peak designed as DE-I which was eluted with (0.12M) phosphate buffer with purification fold 14.67, while the same ion exchanger separated the S-III to two peaks with distinct activity only in one peak which was designated as D-II with purification fold of 13.77.

Optimum pH:

The pH optimum for both DE-I and DE-II' peaks were 6.8 and 7.0. Their pH were equal to that obtained from *P. farcta* fruits [**Sdique**

(1999)]. The results are similar to that obtained from litchi fruit PPO **[Juang (1997)]**.

Table (1): - Purification steps of PPO from leaves of *Prosopis farcta.*

Purification steps	Volume (ml)	Activity (units)	Total Activity (units)	Protein (mg/m)	Specific Activity (units/mg)	(Recovery) Yield %	Fold purification
Extraction with phosphate buffer containing 1% TritonX-100 (Enzyme crude) from acetone powder	60	391	23460	124.2	3.15	100	1
Treatment with Ca-acetate.	50	426	21300	113	3.77	90.7	1.20
Gel filtration on sephadex G-75 (SII)	33	280	9240	27	10.3	39	3.27
Gel filtration on sephadex G-75 (SIII)	47	177	8319	12.6	14.04	35	4.45
Ion-Exchange on DEAE-Sepharose CL6B (DE-I)	27	134	3618	2.9	46.2	15.4	14.67
Ion-Exchange on DEAE-Sepharose CL6B (DE-II')	33	139	4587	3.2	43.4	19.5	13.77

Optimum temperature:

The both enzymes of peaks DE-I and DE-II' show their maximum activity at 45 C° and 40C° respectively. Those results are similar to that formed by Yan **[Yan *et al.*, (1998)]** for lobster PPO.

Effect of divalent activity:

The addition of Cu^{+2} ion exhibit a marked activation of PPO (Table 2). The result of this study is generally similar to that of Juang **[Juang *et al.*, (1997).** This reveals that the Cu^{+2} facilitates the conversion of the apoenzyme into holloenzyme .

Table (2): - Influence of the metal ions on the enzyme activity (*).

The enzyme PPO ac ivity	Salts (0.25mM)
0.132	Fe^{+2}
0.13	Mn^{+2}
0.081	Mg^{+2}
0.15	Ni^{+2}
0.099	Zn^{+2}
0.117	Ca^{+2}
0.157	Cu^{+2}
0.11	None

(*) Activity was determined at 395nm after reaction for 5min. at 35°C and pH 7 with catechol (9.9mM) as a substrate.

Substrate specificity:

The highest activity of PPO was demonstrated as in table 3 with pyrogallol and catechol as substrates, while it had low activity toward m-cresol. This result is similar to that of the litchi PPO which has no cresolase activity **[Juang (1997)]**.

Table (3): - Oxidation of various substrates by (PPO)

Substrate	Activity
m-Cresol	0.092
Catechol	0.326
Pyrogallol	0.464

Separation of β-sitosterol:

The *P.farcta* leaves contain 0.61% β-sitosterol, It was separated by TLC and identified by IR, NMR as in table 4.

Table (4): - Properties of β-sitosterol separated by thin layer chromatography

Solvent	R_f x 100	Colour obtained using 20% H_2SO_4	λ_{max} in CH_3OH (nm)	Infrared data of β-Sitosterol (cm^{-1})			^{1}H-NMR data of β-Sitosterol		
				C=C	C-O	O-H	ppm	Multiplicity	Assignment
Cyclohexan: Acetone: Acetic acid (65: 33: 2)	87	Purple	278, 234	1600	1050	3600-3100(b)	(0.2-3.6) ppm	Multiplet	For $CH_2$and CH_3 protons
Cyclohexan: Ethyl ether: Acetic acid (50: 49.5: 0.5)[11]	60	Purple	278, 234				3.8ppm	Doublet	For OH proton

Separation of flavonoid compounds:

After separation of flavonoids by TLC and paper chromatography three spots corresponding to (myricetin, querciten and kaempferol) were identified and separated using their authentic marker solutions and measuring their spectra as in table (5) .So its worth to mention that this compounds are potent antioxidant and they possess an inhibitory role in various stages of tumor development in animal studies **[Hollman *et al.*, (1997)]**; they also are considered, as anti-inflammatory compounds **[Hollman et al., (2000)]**, and protect the gastric mucosa against variety of ulcerogenenic agents **[Agulio-G (1997)]**. Myricetin and quercetin inhibit both cyclooxygenase and lipooxygenase pathways **[Kim *et al.*, (1998)]**. Myricetin mimics insulin in stimulating lipogenesis and glucose transport in rat adipocytes in vitro **[Ong *et al.*, (1996)]**.

Pectin separation:

P. farcta leaves contains 5.65% pectin, comparing this result with those found in the literature, shows that *P.farcta* leaves contain relatively high amount of pectin, hence the anti-diarrheatic effect of the laves may

be contribute to this amount of pectin which is used as an ingredient in many anti- diarrhea formulation .

Lipid lowering effects of β-sitosterol containing fraction:

Significant drop in serum total cholesterol, LDL, and TG of tested animals were observed at 120 hours, after first administration, while HDL – cholesterol level was increased although it was not significant; these results are indicated in table 6. It's reported that β-*sitosterol* is considered as hypocholestolemic agent, which decreases or blocks the absorption of cholesterol from gastrointestinal tract and by this way lowers the blood cholesterol **[James *et al.*, (1975)]**.

Table (5): - Properties of flavonols, which were separated by paper chromatography

Flavonols	R_f (x 100) in		Colour in U.V. plus ($AlCl_3$/ EtOH)	λ_{max} in CH_3OH (nm)	Infrared data of flavonols (cm^{-1})			
	Forstal	BAW			C=O	C=C	C-O	O-H
Myricetin	28	43	Bright Yellow		1690	1600	1180	3600-2800(b)
Quercetin	41	64	Bright Yellow	253.374				
Kaempferol	55	83	Bright Yellow	268.368				

^{1}H-NMR data of flavonols

Compound	ppm	Multiplicity	Assignment
Myricetin	3.2	M	OH proton
	6.2-8	M	Protons of phenyl rings
Quercetin	3.1	D	OH proton
	6.3-7.9	M	Protons of phenyl rings
Kaempferol	3.4	M	OH proton
	6-7.9	M	Protons of phenyl rings

Solvent key: Forstal = con. HCl: acetic acid: H_2O (3:30:10)
BAW = n. Butanol: acetic acid: H_2O (4:1:5) upper layer.

Table (6): - Statistical analysis of the effect of β-sitosterol on

I- Serum total cholesterol

Cholesterol	Mean mg/100ml	Calculated ±t	Tabulated ±t	Note
Control	89.2	-		
T_1	83	0.472	3.13	N.S.
T_2	71	2.36	3.13	N.S.
T_3	64	3.14	3.13	S. (P<0.01)

II- Low density lipoprotein (LDL-C)

LDL-C	Mean mg/100ml	Calculated ±t	Tabulated ±t	Note
Control	51.6	-		
T_1	46.7	0.48	3.13	N.S.
T_2	34.8	2.75	3.13	N.S.
T_3	28.8	5.25	3.13	S. (P<0.01)

III- Triglyceride

T.G	Mean mg/100ml	Calculated ±t	Tabulated ±t	Note
Control	74.5	-		
T_1	64.2	1.08	3.13	N.S.
T_2	48.8	1.8	3.13	N.S.
T_3	37.4	7.24	3.13	**S. (P<0.01)**

IV- High density lipoprotein (HDL-C)

HDL-C	Mean mg/100ml	Calculate ±t	±t tab 0.01	Note
Control	22.4	-		
T_1	24.3	0.46	3.13	N.S.
T_2	26.5	0.65	3.13	N.S.
T_3	28.1	1.63	3.13	N.S.

T_1 = 48 hrs. after administration (2 days)
T_2 = 96 hrs. after administration (4days)
T_3 = 120hrs. after administration (6 days)

REFRENCES

Agullo G; Biochem-Phermacol., 1, 53(11), 1649-1657, Jun., (1997).

Finar I.L., Organic Chemistry , Vol.2, 5th ed. Longman, (1975).

Goodwin T.W E Chemistry and Biochemistry of Plants, 2, Acadimic press (London), (1976).

Husni A. Twaij, A J.Biol. Sci, 14(1), 59-68, (1983).

Hollman Pc;.Katan MB. Biomed-Pharmacother. 51(8), 305-310, (1997).

Harborn J.B Phytochemical Methods. 12ed. London, Newyork.Chapinan and Hall, , (1984).

Harborn J.B and Swan T. Perspectives in Phytochemistry, Academic press, London., (1969).

Hollman PCH, Arts ICW. Flavones and Flavanols nature, occurrence and dietary burden. J Food Sci Agri 80:1081-93(2000)

Ikan R and. Cudizinoviski N. J. Chromatography , 18, 419-421,(1965).

Jayaraman K. S,. and Ramanuja M.N. Vijayaraghavan, P. K. Food Chemistry, 24,203-217, (1987).

James M and.Ottow O ,Human Biochemistry,9th ed.,(1975)

Karting TH and. Mikula G, J. Chromatog.,53, 537-543, (1970).

KimHp,.Mani;.LversenL;.ZibohVA;.Prostaglands-leukot-Essent-Fatty-Acids,58(1),17-24Jan.,(1998) .

Lowry O.H. Roberough, N.J, A.L. Farr and R.J Randall. J, Biol, chem., 193, 265, (1951).

Mayer;.Harel E:Phytochem.5 .783(1966).

Ong Kc;.Khoo He;Bio. Pharmacol.235(4),423-429(1996).

Prabha T.N and. Patwardhan M. V. Journal of Food Science and Technology, 22,404-407,(1984).

Senatore-AT; Watkins-WD; Nguyen-TD. Toxicol-Appl-Pharmacol.15,99(2): 357-361, Jun , (1989).

Sdique M, MSc. Theases, Univ. of Salahaddin, Erbil, (1999).

Townest C. and.Guest E.Flora of Iraq. Part I,(1974).

Venkaiah B and. Palwardhan M.V.Food Science and Technology,14,174-175(1977).

Yan X,.Taylor K.D.A and.Hanson S.W.J.food chemistry 36, 19-30(1990).

فصل البوليفينول أوكسيديز والتعرف على بعض المركبات ذات النشاط البيولوجى فى أوراق بروسبس فركتا

تستخدم البروسبس فركتا من عشرات السنوات فى الطب الشعبى لمعالجة عديد من الأمراض التى تشمل مرض السكر – الإسهال – زيادة اللبيدات والمغص المعوى. ولهذا السبب تم فصل العديد من المركبات الفينولية والتعرف عليها. كما تم فصل إنزيم البولى فينول أوكسيديز والذى يلعب دورا هاما فى تكوين عديد الفينولات وتم التعرف على الإنزيم بالطرق الكروماتوجرافية.

هذا وقد تم الفصل والتعرف على المركبات ذات النشاط البيولوجى مثل الميرستين وكيروستين والكامفيرول والتى لها أثر واضح كمضادات للأكسدة ومعادلة الشقوق الحرة – وقد إتضح أن مركب البيتاسيسترول له أثر مضاد للإلتهاب وخفض اللبيدات منخفضة الكثافة فى الدم وتم فصل هذا المركب بإستخدام طرق اسبكتروسكوبية مختلفة.