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ABSTRACT

Boronic acids monomers shows optimum adsorption of diols at pH 8.5. In this study, we were able to reduce this pH to 7 by using 4-(*N*-allylsulfamoil)phenylboronic acid (ASPBA) monomer copolymer. Methacrylate (MA) and our synthesized ASPBA were used to prepare millimeter sized poly[methacrylate-*co*-4-(*N*-allylsulfamoil) phenylboronic acid] (poly[MA-*co*-ASPBA]) particles. These particles were characterized by ^{1}HNMR, FTIR and TGA. The prepared particles were mixed with the extract of *Hypericum perforatum L.* (centaury) and showed highly selective interaction with the flavonoids. Antioxidant and antiradical compounds were adsorbed over the surface of the particles and later desorbed. These macroporous particles can be used in selective isolation of natural products from plant extracts, especially cis-diols. Our prepared particles can be used as packing materials in the columns to selectively capture the target polyphenolics. Alternatively, the particles can be mixed with the extracts to adsorb the target polyphenolics, and then desorb to obtain selective isolation.

Keywords: Boronate affinity, sulfonyl and sulfonamide affinity, flavonoids, polymeric adsorbent, adsorption, purifications

INTRODUCTION

Flavonoids are used in various medical and cosmetic applications due to their biological and physiological activities e.g. anti-inflammatory, antitumor, antioxidant and radical scavenger. Due to these important bioactivities, flavonoids are most important group of the natural products that are under investigation (HAVSTEEN, 2002, WALLE, 2004).

Various studies performed on *Hypericum perforatum L.* (centaury) have shown its antidepresant, anti-anxiety, antiviral, and antimicrobial activities (BARNES, 2001; BUTTERWECK, 1997, 2000, 2002; FLAUSINO, 2002; SAKAR, 1990). A formulation (LI 160) prepared from *H. perforatum* extract has showed psychvegetative and anti-depressive activities in 12 years' age group (HUBNER, 2001). Various flavonoids (CHUNG, 1997; DIAS, 1998; ISHIGURO, 1991, 1993), xanthons (GUNATILAKA, 1979; RATH, 1996; WU, 1998), chromenyl ketones (ISHIGURO, 1990; WU, 1998), hyperphorines (DECOSTERD, 1989; MAİSENBACHER, 1992; TRİFUNOVİC, 1998), phyloroglusinoles (DECOSTERD, 1991; ISHIGURO, 1998; ISHIGURO, 1994), n-alkanes (BRONDZ, 1983) naphthodiahthrones (KITANOV, 2001) volatile oils (CAKIR, 1997) and biflavonoides (CAKIR, 2003) has been reported from *H. perforatum*.

The aqueous alcoholic extract (60 % ethanol or 80 % methanol) obtained from aerial parts of *H. pericum perforatum* plant consists of various groups of natural products (SADDİQE, 2010). These are naphthodianthrones (hiperisin and pseudohipersin), phyloroglucinoles (hyperphorines and adhyperphorines), flavonoides (kaempferol, luteolin, mirisetin and kersetin), hyperosite (hiperin), rutin, kercitrine and isokercitrine), biflavones (biapigenin, amentoflavone, diquercetin), phenylpropane (p-coumaric acid and cafeic acid, chlorogenic asit), volatile oils (aliphatic compunds eig. 2-methyl octane, n-nonane, n-decane, n-undecane, n-tetradecanol, 2-methyl-decane and 2-methyl-dodecane) and terpenoids (α-pinen, β-pinene, geraniol, β-caryophylene, β-farnecene, humulen and germacrene D). Additionally tanines, xanthones, essential oils and amino acids are also found in less amounts. Some important phenolics are given in Figure 1.

Among the extraction and purification procedures of natural flavonoids, solvent extraction is very old and developed method (HE, 2002; TAKEDA, 1988). However, due to the increasing environmental pollution and waste produced in the result of solvent extraction, there is a need of environmental friendly, highly selective isolator, high yield and less toxic techniques. There are few reports of using macroporous resins to gain these purposes (PI, 2008). There are few reports of using polymeric adsorbents to enrich flavonoids (XU, 2000; FU, 2005; MARIA, 2002; SCORDINO, 2003: ZHANG, 2007).

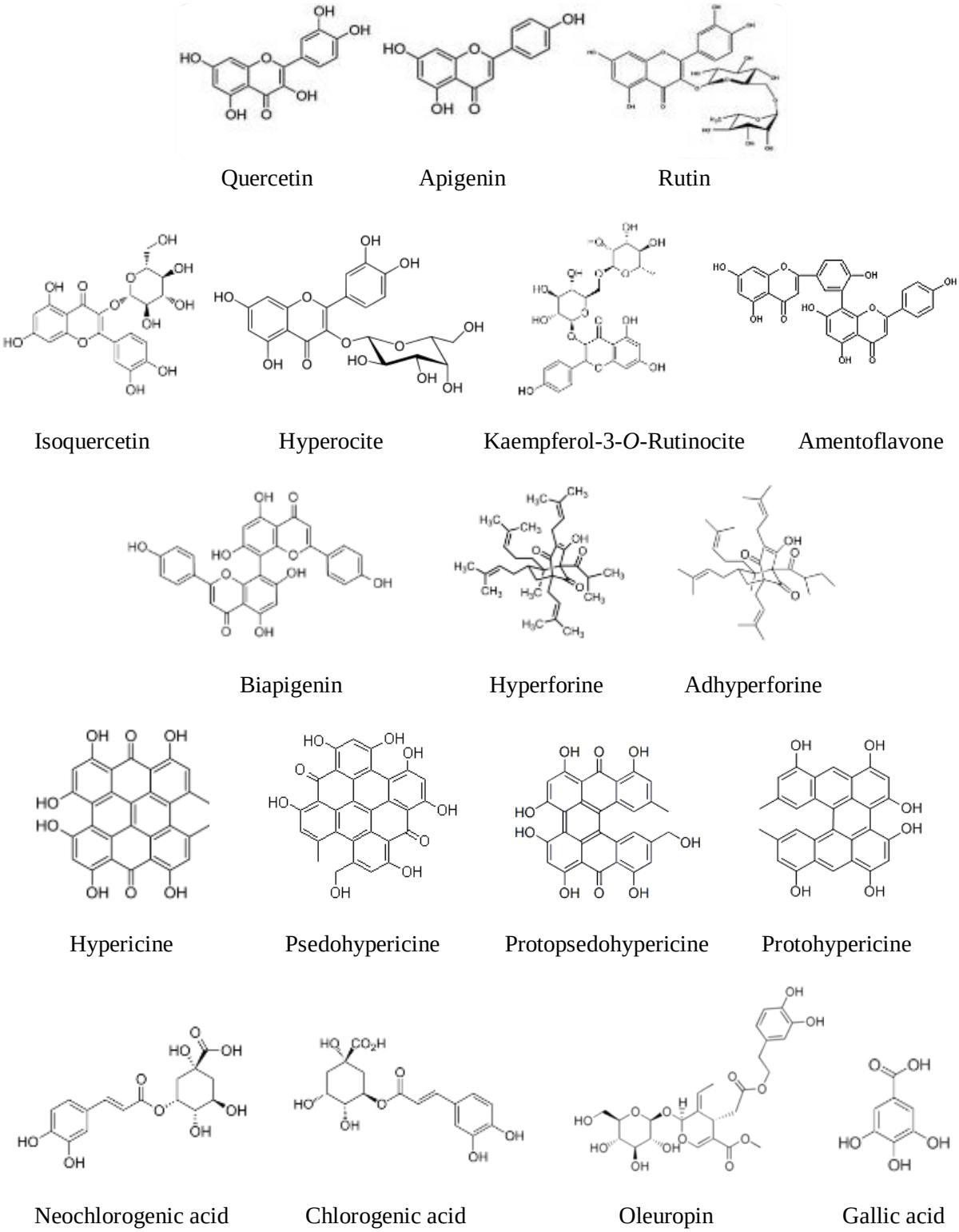

Figure 1. Quercetin, apigenin, rutin and other flavonoids from *H. perforatum* tested in the adsorption studies.

Compounds given in Figure 1 have been isolated from alcohol and acetone fractions as major compounds. There is a need to increase the amount of these hydroxy containing compounds. To separate these type of compounds, there was a thought to utilize boronic acid compounds due to their attraction towards hydroxl and diol compounds.

Boronic acid-based materials used for chromatographic purposes falls under "boronate affinity chromatography". In this type of affinity, boronates group creates a complex with the target molecules (SENEL, 2002). To purify nucleotides, RNA, glycated proteins and glycoenzymes, particles having boronic acids are used in the column. For this purpose, functionalized polyacrylamide, agarose and polyacrylates-based jells are preferred.

In this study, we have utilized the functionalized molecules that are very like the ones synthesized by Geng et al. (GENG, 2009). The difference is that our molecules possess hydroxy loving boronic acid groups. A positive side of this study is that, our adsorbent materials are vary suitable to perform adsorption at neutral pH instead of pH 8.5. Flavonoids were isolated from ethyl acetate (EtOAc) fraction of *H. perforatum* by using our synthesized poly(MA-*co*-ASPBA) suspended macroparticles that were prepared from boronic acid containing ASPBA monomer. Antioxidant and antiradical activities of these flavonoids were also tested. As a reference compound, quercetin that contains neighboring hydroxyls was used in the adsorption studies.

RESULTS & DISCUSSIONS

Isolation of flavonoids from *H. perforatum* extracts by millimeter-sized poly(MA) homopolymer and poly(MA-*co*-ASPBA) particles

In this part, first ASPBA (**1**) was synthesized and characterized. FTIR spectra of the synthesized ASPBA monomer and its precursor *N*-allyl-4-bromobenzensulfonamide (**2**) are given in Figure 2 (A and B). In Figure 2A, characteristic bands related to amine and double bonds, and in Figure 2B, red encircled band related to boronic acid moiety shows the successful synthesis of ASPBA.

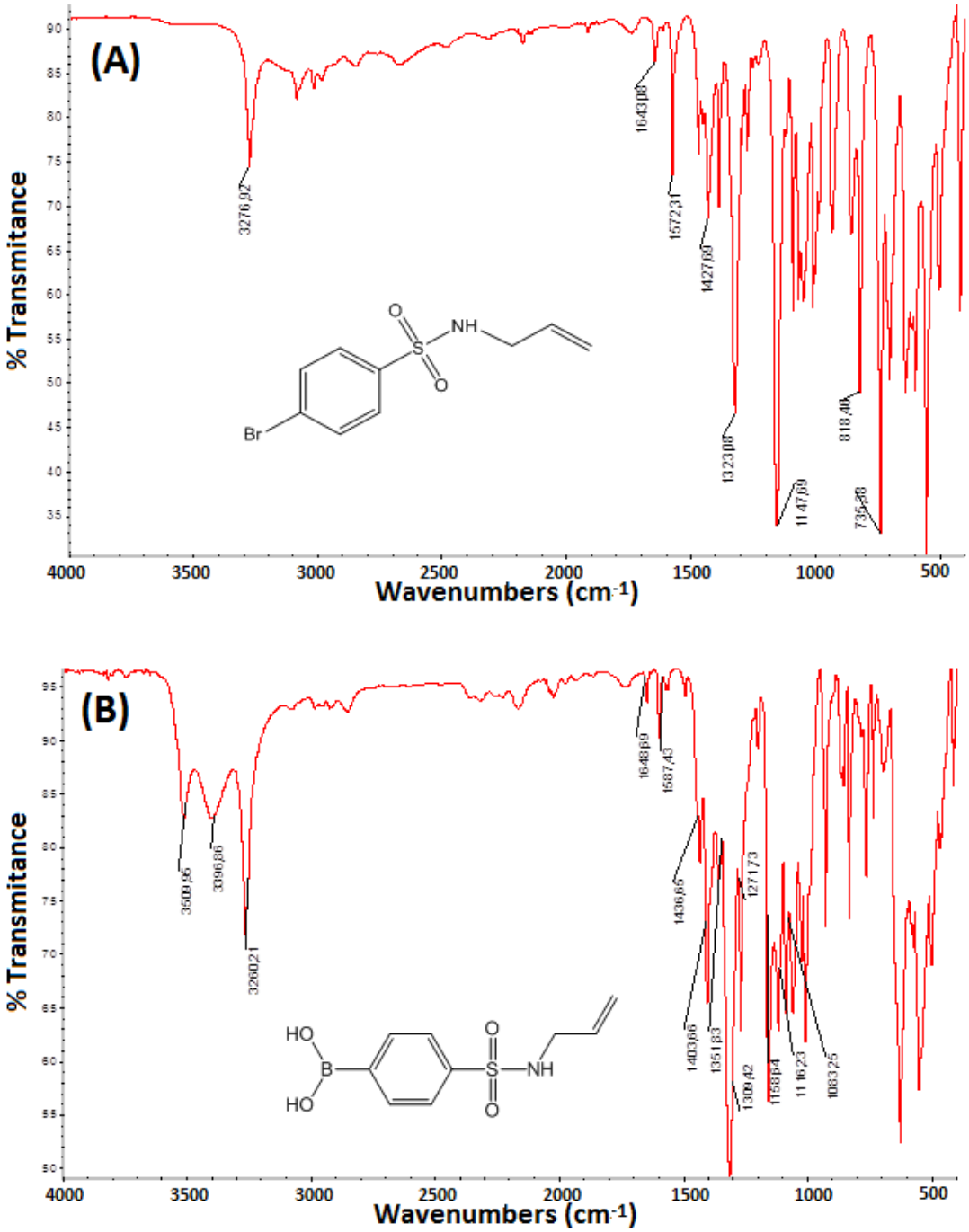

Figure 2: FTIR spectra of *N*-allyl-4-bromobenzensulfonamide **(A)** and ASPBA **(B)**

^{1}HNMR of ASPBA (**1**) monomer and its precursor *N*-allyl-4-bromobenzensulfonamide (**2**) are given in Figure 3 (A and B). The peak related to the solvent DMSO-d_6 is pointed out with "x" in the ^{1}HNMR spectrum of **2**. The peaks encircled in yellow are related to the impurities while the peak related to -NH- is pointed out with "d". In the right inset of Figure 3, molecular structure is shown with numbered protons and their peaks are shown on the left inset of the Figure (LI, 2008). The peak shown with "g" is related to the diols of boronic acid. The spectra show that ASPBA has been synthesized successfully.

The synthesized ASPBA monomer complexed with the monomer MA utilizing its cis diols at neutral pH (7.0) instead of pH 8.5. Also, they showed high antioxidant activities and high adsorption properties that increased the excitation in this study. For this purpose, first MA/ASPBA complex was prepared with different formulations that are given in Table 1.

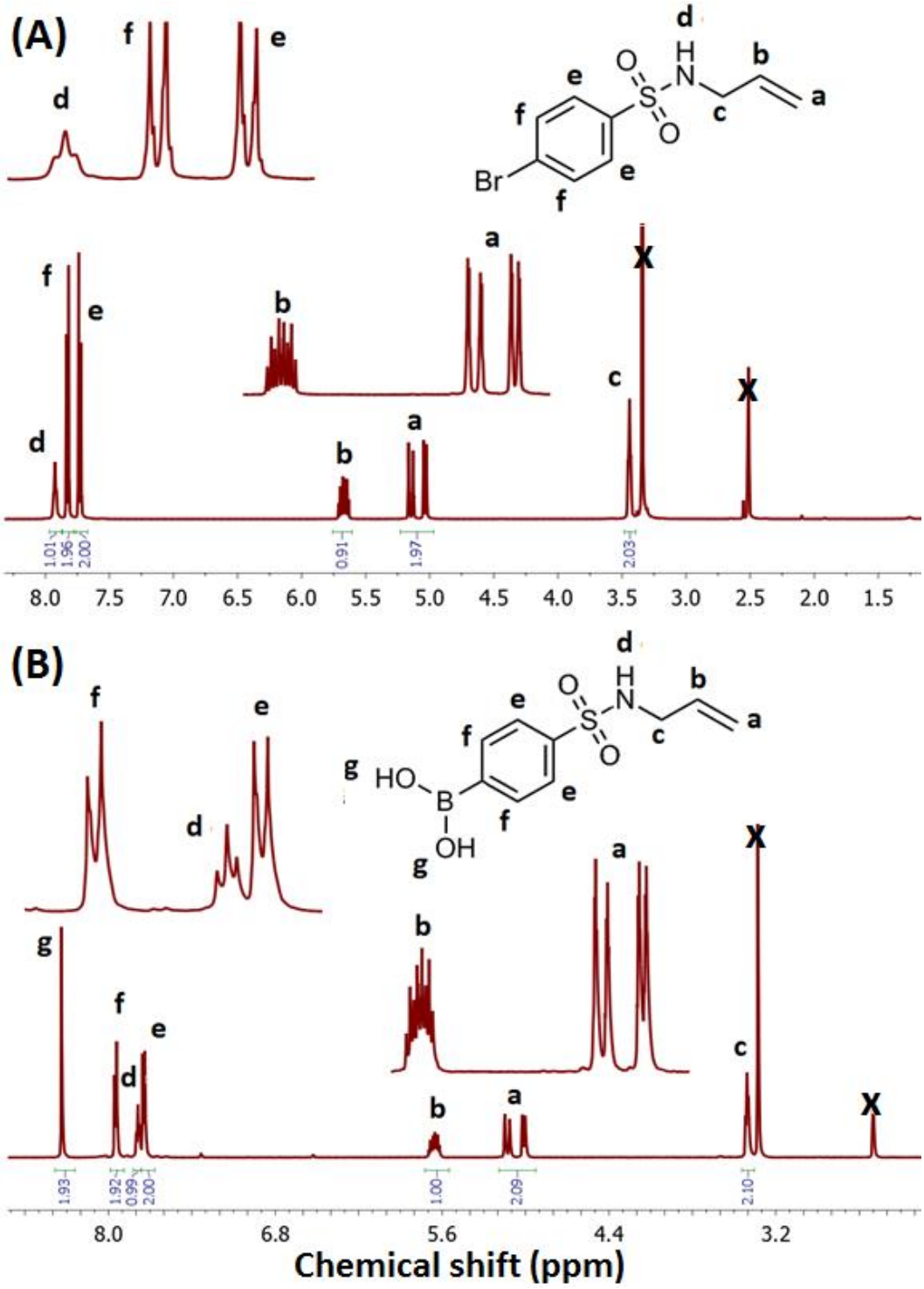

Figure 3: [1]HNMR spectra of *N*-allyl-4-bromobenzensulfonamide **(A)** and ASPBA **(B)** (in DMSO-d_6)

.

Table 1: Various formulations to synthesize poly(MA-*co*-ASPBA)

Sample	PVP (%)	PVA (%)	Distribution medium (mL)	Methacrylate (mL)	EGDMA (mL)	ASPBA (g)	BPO (g)	AIBN (g)	Methoxy ethanol (mL)	Dodecanol (mL)	Dibutyl phthalate (mL)	Ethanol (mL)	Butyl Acetate (mL)	Dioxane (mL)	DMSO (mL)	salt (w/v %)
SP-61	1	…	8	1.58	0.3	0.64	0.05	…	…	0.60	0.7	…	0.12	0.2	0.12	…
SP-62	1	…	8	1.58	0.3	0.96	0.06	…	…	0.70	0.6	…	0.12	0.2	0.24	…
SP-63	1	…	8	0.94	0.3	…	0.02	…	0.2	0.66	0.18	…	0.60	0.3	…	…
SP-65	1	…	8	1.26	023	1.03	0.06	…	…	0.60	1.6	…	0.12	0.2	0.42	…
SP-66	1	…	8	1.58	0.3	…	0.05		…	0.60	0.6		0.12	0.2	0.36	…
SP-67	1	…	8	1.58	0.3	…	0.05		…	0.60	0.6		0.12	0.2	0.36	2.5
SP-68	1	…	8	1.26	0.23	1.03	0.06	…	…	0.60	0.6	…	0.12	0.2	0.42	2.5

Unlikely to our expectations, no spherical particles were formed after complexation of ASPBA with MA as a result of formulations given in Table 1. Prepared particles were of 1.-3 mm in size. To obtain spherical particles, the amount of salt was changed in the distribution medium but in vain. No matter the shape of the particles, poly(MA-*co*-ASPBA) was characterized. Photographs of the particles obtained from various formulations i.e. SP-61, SP-62, SP-65 and SP-66 are given in figure 4. Similarly, photos of particles obtained without salt in the polymerization medium i.e. SP-67 and SP-68 are also given.

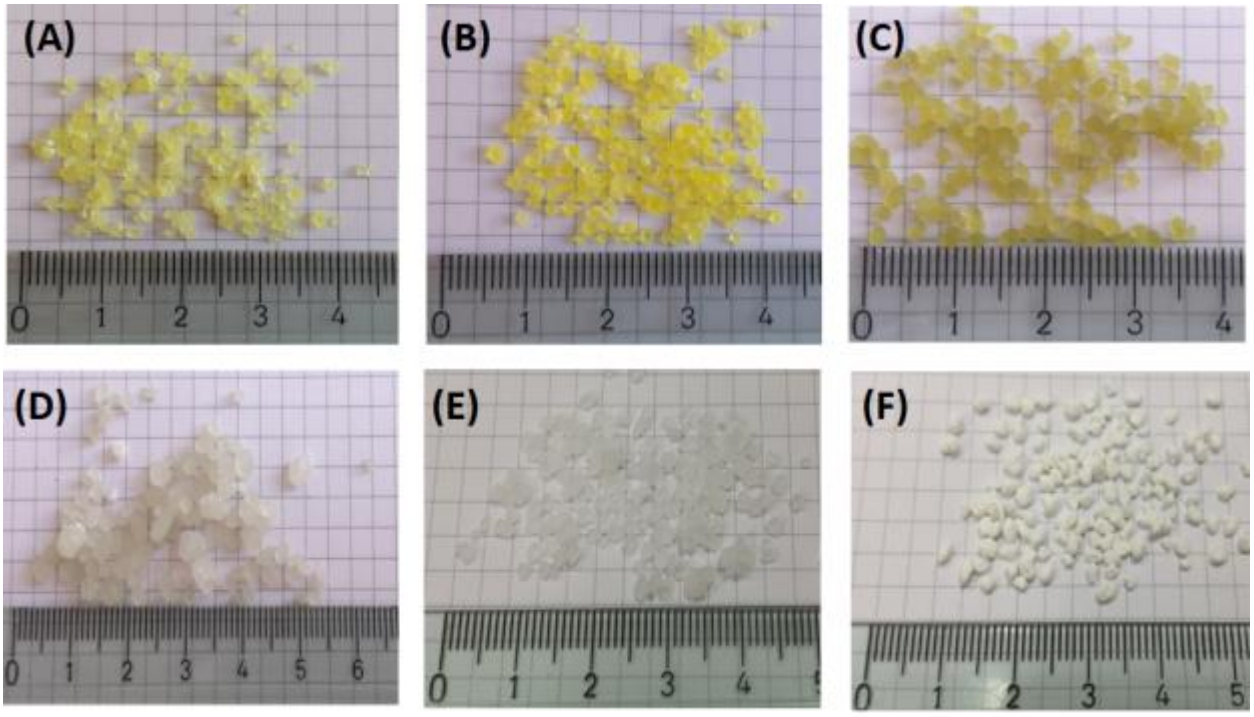

Figure 4: Photographs of the prepared macroparticles (A): SP-61, (B): SP-62, (C): SP-65, (D): SP-66, (E): SP-67, (F): SP-68

An increase of 1 to 2.5 mm was observed in the poly(MA-*co*-ASPBA) particles when the amount of ASPBA was increased as show in the samples SP-61, SP-62 and SP-65. On the other hand, particle size was decreased when the salt was added in the polymerization. This can be observed when SP-65 is compared with the SP-68.

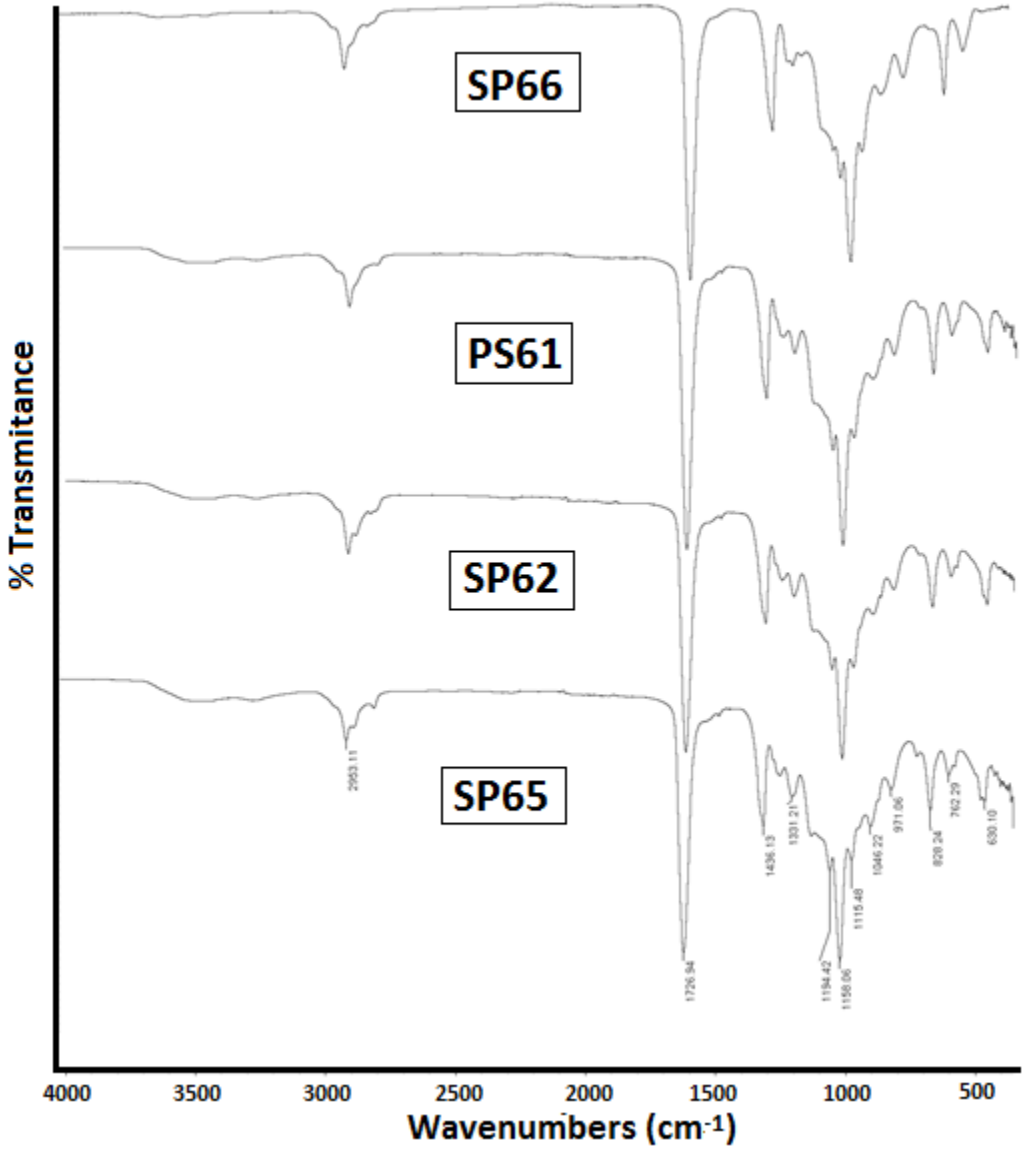

Figure 5: FTIR spectra of poly(MA-*co*-ASPBA) and poly(MA) prepared with various MA/ASPBA copolymerization input amounts. Copolymerization feed ratio of MA/ASPBA (mL g^{-1}): 0 (SP-66), 0.64/1.58 (SP-61), 0.96/1.58(SP-62) and 1.03/1.26(SP-65).

FTIR spectra of the prepared polymeric particles are given in Figure 5. As it is clear from the spectrum, the bands at 3500 and 3300 cm^{-1} that are not present in the spectrum of poly(MA) (SP-66) were appeared in poly(MA-*co*-ASPBA) due to ASPBA from the cis diols connected to boron.

Another band was also spread and 1331 cm^{-1} that can be related to R-SO_2-NH. However, the intensity of the band at 1330 cm^{-1} due to the presence of ASPBA in the co-polymer is the same in the samples SP-61, SP-62 and SP65. The percent amount of ASPBA in the copolymer is 49, 40 and 54 as was found in the TGA analysis (Table 2); those are very near to each other. 10 % loss of SP-66 occurred at 380 ^oC while it happened at 360 ^oC in pure ASPBA. As the amount of ASPBA increased in the copolymer, the decomposition temperature became near to the decomposition temperature of the pure ASPBA.

After the addition of ASPBA to the copolymer, the copolymerization yield decreased from 70 to 35 % (Table 2). The copolymerization of high hydrophilic ASPBA momoner and more hydrophobic MA monomer has decreased the polymerization yield, also shapeless particles were prepared instead of spherical particles. Allylic effect decreases the polymerization (SEPEHRİANAZAR, 2008), and that happened in our case also.

Table 2: Unburned boron after TGA analysis, and the decomposition temperature at 10 % weight loss of ASPBA in the copolymer

Sample	Polymerization % yield	Monomer feed ratio of ASPBA/AM (g mL^{-1}) at initial polymerization	Copolymer ASPBA percentage (%, g/g) in polymer	10 % weight loss copolymer decomposition temperature (^oC)
SP-61	36.5	0.64/1.58	49	380
SP-62	34.8	0.96/1.58	49	370
SP-65	33	1.03/1.26	54	375
SP-66	71	0*	0	395
ASPBA	-	-	-	360

The adsorption studies over the prepared copolymer were performed on quercetin at both 7.2 and 8.5 pH to test the effects of cis-diols. Table 3 shows the adsorption capacity results of the ASPBA containing copolymer. The percentage of quercetin adsorption in SP-61, SP-62 and SP-65 at pH 7.2 was very close to the that of adsorbed at pH 8.5. Samples SP-66 and SP-67, which do not

contain ASPBA, do 20-40 % less adsorption as compared to SP-61, SP-62 and SP-65 but the adsorption capacity did not change when the pH was changed.

Table 3: Variations in the percentage of ASPBA/AM ratio (g mL^{-1}) with quercetin adsorption

	Polymerization monomer feed ratio of ASPBA/AM (g mL^{-1})	Copolymer ASPBA (%) (g/g)	Quercetin adsorption capacity (mg Quercetin/g particles)	
			pH 7.2	pH 8.5
SP-61	0.64/1.58	49	0.161	0.191
SP-62	0.96/1.58	49	0.150	0.176
SP-65	1.03/1.26	54	0.087	0.095
SP-68	1.03/1.26*	34	0.131	0.156
SP-66	0*	0	0.030	0.031
SP-67	0**	0	0.060	0.052

* Use of salt in the copolymerization distribution medium
** Use of 2.5 % salt in the copolymerization distribution medium

Particles prepared by formulations SP-65 and SP-66 having similar particle diameters. These formulations has 4 times more adsorption capacity as compared to SP-66 and SP-67 homopolymers. New particles prepared from poly(MA-*co*-ASPBA) can compete with the adsorption at 8.5 pH. Additionally, they can also show the higher adsorption capacity that is shown by poly(MA) alone. The question is, how to utilize this adsorption capacity over plant extracts? The answer is hidden in the table 5 and 6 that shows the antioxidant activity and DPPH remedy.

As seen from tables 4 and 5, among EtOAc extract, Ads (remains from adsorption) and Desp (desorbed), Desp showed the highest antioxidant and antiradical activities. It supports our previously reported results where the adsorption was performed at pH 8.5 by vinylphenylboronic acid (ÇETİNKAYA, 2012).

Table 4: Antioxidant activity of poly(MA-*co*-ASPBA) particles obtained by β-Carotene

		Antioxidant Activity		
		100 ppm	200 ppm	350 ppm
EtOAc extract		61.3 (±0.7)	67.0 (0.2)	75.3 (±0.5)
SP65	Ads.	5.0 (±0.3)	11.8 (±1.8)	20.3 (±2.1)
	Desp.	38.5 (±0.5)	89.7 (±1.9)	100.0 (±3.0)
SP66	Ads.	1.2 (±0.2)	3.4 (2.4)	39.5 (±1.7)
	Desp.	9.9 (±2.9)	46.8 (1.6)	64.9 (±1.4)

Table 5. Antioxidant activity of poly(MA-*co*-ASPBA) particles obtained DPPH radical scavenging activity

		Antiradical Activity (DPPH scavenging)		
		100 ppm	200 ppm	350 ppm
EtOAc extract		29.4 (±2.7)	40.9 (±6.2)	63.9 (±0.4)
SP65	Ads.	11.3 (±0.8)	18.6 (±0.6)	31.5 (±1.0)
	Desp.	41.6(±0.8)	76.3 (±0.7)	100.0 (±1.1)
SP66	Ads.	26.2 (±0.6)	47.9 (±1.1)	77.1 (±1.1)
	Desp.	8.2 (±1.8)	21.0 (±0.49)	31.1 (±2.3)

EXPERIMENTAL

The target macroparticles were prepared by suspension polymerization. Methyl acrylate (MA), 2,2′-azobis(2-methylpropionitrile) (AIBN) starter (Acros Organics, New Jersey, USA) or benzoyl peroxide; polyvinyl alcohol (PVA, MW 85,000-146,000, %87-89 hydrolyze form) or polyvinylpyrrolidone (PVP K-30, M_n:40.000 g mol^{-1}, Sigma Chemical Co., St. Louis, MO) as stabilizer; ethylene glycol dimethacrylate and divinyl benzene (DVB, %55 para- and meta divinylbenzene isomer containing, Aldrich Chem. Co.) as cross-linker were used. Separately, heptane, *n*-butyl acetate, ethyl acetate (EtOAc, % 99.5) dodecanol, dibutylphthalate (DBP, Aldrich Chem. Co.) n-butyl phthalate, dioxane, dimethyl sulfoxide and 2-methoxyethanol was used as pore formers. Ionic balance was provided by using sodium chloride (NaCl). All

polymerizations were performed in distilled water. 4-Bromobenzensulfonyl chloride, allylamine, triisopropyl borate, acetonitrile (CH_3CN)**,** 1.6 M *N*-butyllithium (*n*-BuLi, in hexane), *N*-methyl-diethanolamine 4-(*N*-allylsulfamoil) phenylboronic acid (ASPBA) monomers were used in the synthesis. Quercetin hydrate was used as a model flavonoid in the adsorption studies (> %95, Aldrich). Antioxidant activities and total flavonoids of plant extracts was performed by *β*-karoten (Fluka, 97 %), linoleic acid (Aldrich, 99 %), 1,1-diphenyl-2-picrilhydrazil (DPPH) (Fluka, 85 %), and Tween-40 (Merck). All solvents used were of analytical grade.

Synthesis of Macroporous poly(MA), the starter particles

Poly(MA) starting particles were prepared by suspension polymerization method (GENG, 2009). Monomer phase 5.44 mL MA monomer, 0.96 mL EGDMA cross-linker monomer, 0.12 g AIBN radical initiator, pore formers (0.8 mL heptane, 0.8 mL n-butyl acetate mixture) were mixed and transferred into 250 mL round bottom flask. To this mixture, 2 % NaCl containing 80 mL % 0.5 (g/g) PVA was added and stirred at 250 rpm. The mixture was first heated at 68 ^oC for 4 hours, then 88 ^oC for 6 hours. Filtered particles were washed with excess hot water, then ethanol. At last, washed for 3 days with soxhlet apparatus with dried petroleum ether to produce pores.

Synthesis of monomer 4-(*N*-Allylsulfamoil)phenylboronic acid (ASPBA)

First, ASPBA co-monomer was prepared in the lab to utilize in the synthesis of macroporous poly(MA-*co*-ASPBA) particles. ASPBA monomer was synthesized according to the literature procedure (LI, 2008) (Figure 6). To a solution of 4-bromobenzenesulphonyl chloride (12.8 g, 50 mmol) and 5 mL triethylamine in 100 mL of acetonitrile under nitrogen environment and salt-ice bath at -10 ^oC, 10 mL allylamine dissolved in 10 mL acetonitrile was added dropwise. The temperature did not exceed 0 ^oC during the addition. After the addition, the ice bath was removed and the mixture was stirred under nitrogen overnight. Acetonitrile was evaporated under vacuum after the reaction, and the remaining mixture was dissolved in 50 mL chloroform. Organic phase was washed with saturated $NaHCO_3$ (4x50 mL), then with 50 % NaCl (2 x 30 mL), then dried over $MgSO_4$. Solvent was evaporated that left a light-yellow matter behind. The compound was crystallized by aqueous ethanol.

In the second step, in a dried mixture of THF and toluene (4/1 v/v) in a 100 mL round bottom flask under argon environment and dry ice/acetone bath, 11.0 g N-allyl-4-bromobenzenesulphonamide and triisopropyl borate (48 mL, 0.2 mol) were added slowly. 1.6 M *n*-butyllithium (100 mL, 0.16 mol) was slowly added by a dropping funnel during 1 hour at -78 ºC. The mixture was stirred at -78 ºC for 1 hour, then left overnight to reach slowly at room temperature. After finishing the reaction, 100 mL water was added to the reaction mixture and stirred for 30 minutes. pH was adjusted to 6.5 by using 2 M HCl. Aqueous phase was extracted by chloroform and dried over $MgSO_4$. Solvent was evaporated to obtain the target boronic acid product. The prepared products were characterized by FTIR and NMR.

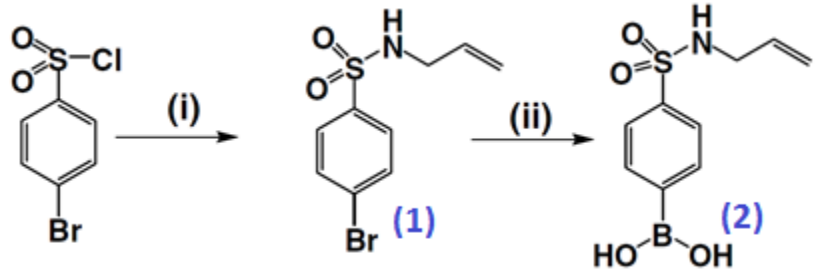

Figure 6: Synthesis of 4-(*N*-allylsulfamoil)phenylboronic acid monomer: (i) allylamine, TEA, CAN; (ii) triisopropyl borate/n-butyllithium, THF/toluene (1:4, v/v), -78 ºC

Synthesis of macroporous millimeter-sized poly(MA) and poly(MA-*co*-ASPBA) particles

Poly(MA) and poly(MA-*co*-ASPBA) were prepared by suspension polymerization by heating magnetic stirrer in a glass reactor. In a typical co-polymerization reaction, 1.5 mL MA, 0.3 mL EGDMA, 0.04 g BPO and 0.94 g ASPBA were mixed in a glass tube. In this small glass tube 1 % (w/v) PVP was solved and the mixture was stirred at 400 rpm. To this mixture, organic phase prepared in the previous step was added, sealed, heated first at 82 ºC for 4 hours, then to 90 ºC for one hour and quenched the reaction. The prepared particles were washed with hot water, ethanol, dimethyl sulfoxide (DMSO), ethanol and again with water, then, left at fuming hood to dry. After drying, the prepared particles were subjected to yield calculation of the monomers by gravimetric analysis, while the structure was characterized by FTIR and TGA.

Structural characterization and analysis

All compounds synthesized during the preparation of ASPBA monomer were characterized by NMR. Compounds were analyzed in DMSO-d_6 over Bruker 400 MHz Avance NMR instrument. FTIR of the compounds was performed on ATR FT-IR Perkin Elmer. Washed homo and copolymeric particles were dried in the fuming hood, then forwarded to FTIR to analyze amine and boronic acid. TGA of all particles were tested at 40-600 ^oC temperature with 20 ^oC min^{-1} heating using Perkin Elmer 4000 model TGA under nitrogen atmosphere.

Model Flavonoid Studies

The model flavonoid i.e. quercetin was adsorbed over the prepared particles. Schematic representation of the adsorption study is given in Figure 7. HEPES/methanol buffer was used to adjust pH 7.2 and 8.5. A 0.02 mg mL^{-1} of quercetin solution was prepared for adsorption studies. 10 mL of quercetin solution mixed with certain number of particles for 90 minutes. Initial and final absorbance of the solution was obtained at 374 nm by UV-visible spectrophotometer. Adsorption capacity was calculated using the following equation (eq. 1):

$$\text{Adsorption capacity} = [((A_o\text{-}As)/A_o) \times C_o \times V]/ W_d \qquad (1)$$

where, A_o, A_s, C_o, V and W_d are the initial absorbance of quercetin, absorbance of quercetin after 90 minutes, initial concentration of quercetin, volume of quercetin of adsorption medium and dry weight of the polymeric particles used. Figure 7 shows the adsorption mechanism of the flavonoids over the particles.

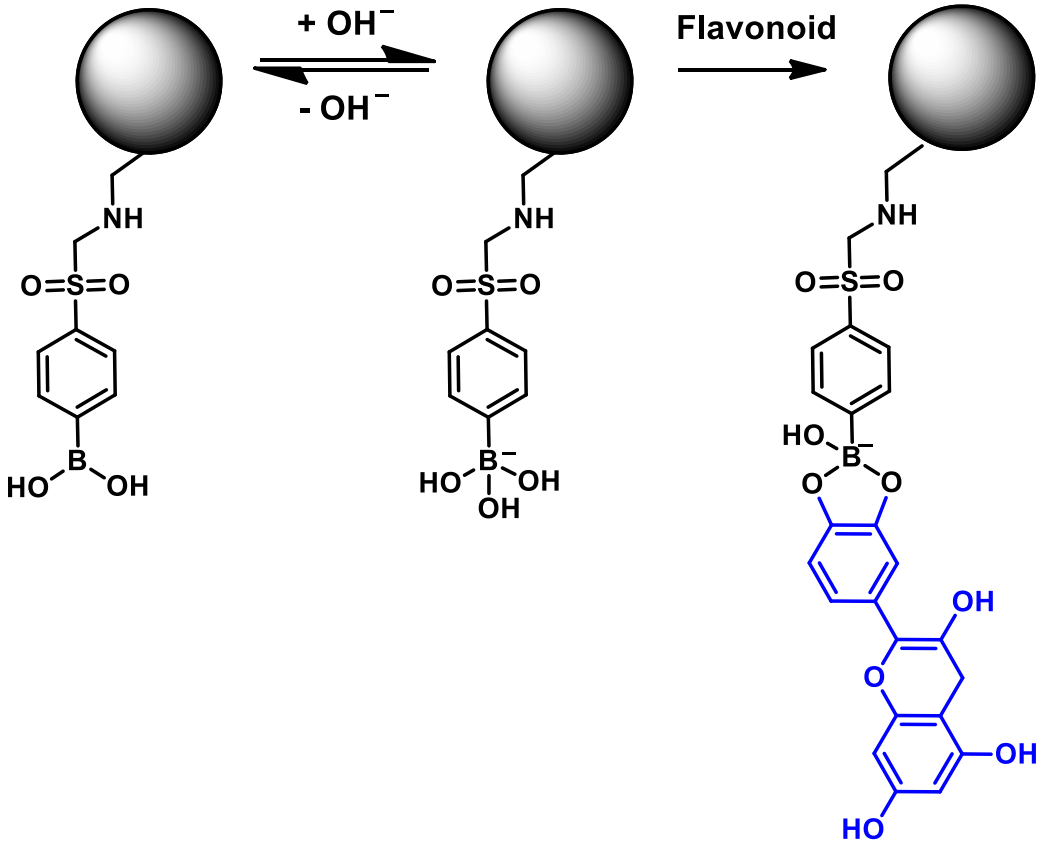

Figure 7. Schematic representation of flavonoid adsorption on the ASPBA containing particles

Using of poly(CMS-*co*-DVB) microspheres for antioxidant activity increments, preparation of ethanol and ethyl acetate extracts, adsorption from ethanol and ethyl acetate extracts, and calculation of desorption percentage, free radical scavenging activity (DPPH method), determination of antioxidant activity with *β*-carotene-linoleic acid assay are discussed in detail in our previous study (Onur, 2012)

CONCLUSIONS

Plant extracts are enough sensitive to pH. Certain special compounds can be isolated from the plant extracts by adsorption at neutral pH. Most of the boronic acid-based monomers having pK_a of about 8.0 and above. The pK_a value of ASPBA is about neutral pH, that is why this moiety has been used in this study. We have successfully adsorbed cis-diols from the plant extract over the

poly(MA-*co*-ASPBA) copolymer particles at neutral pH. The adsorbed compounds were back desorbed and were found as highly antioxidant and antiradical active. This is the first report of isolation of bioactive cis-diols from *Hypericum perforatum L.* plant extract by our synthesized polymeric particles at neutral pH. These types of particles can be produced at commercial level and used in pharmaceutical industries. Additionally, there is also a need to prepare other polymeric particle to be used in selective isolation of steroids, alkaloids, terpenes etc.

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