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INVESTIGATIONS ON RAPARIN OBTAINED FROM RAPANA VENOSA (VALENCIENNES)

RAPANA VENOSA (VALESIENNES)'DAN ELDE EDİLEN RAPARİN ÜZERİNDE ÇALIŞMALAR

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Key words : Raparin, fractionation, biological activity assay, recalcification time, metachromatic activity

Abstract

Heparin was first isolated in 1916 from animal tissues. Raparin, heparin like substances, was isolated from *Rapana venosa* (Valenciennes) in 1991. In this study, raparin was extracted from hepatopankreas of *Rapana venosa* by three different techniques and fractionated through Sephadex G-50 Super fine column. Two fractions were obtained and their recalcification time, Heptest, Hepaclot methods and metachromatic activity were studied. All fractions showed activity in recalcification time assay and Raparin RKL_{10} Fr I showed the greatest activity. All fractions were showed activity in Heptest but only raparin RHP_{10} Fr II demonstrated considerable activity with Hepaclot test. Raparin RH_{500} Fr I showed metachromatic activity with azure A and toluidine blue dyes.

Introduction

Heparin was the first to isolate from dog liver which retarded the coagulation of blood in vitro (Mc Lean 1916, Howell and Holt 1918). Heparin has been a widely used as an anticoagulant drug indicated for the prophylaxis and treatment of thrombotic disorders.

Numerous anticoagulant methods have been developed to asses of the potency. Heparin is determined generally by biological and less frequently by metachromatic methods. Metachromatic assay of heparin was proposed (MacIntosh 1941, Jaques *et al.* 1947) and correlation between the methods were investigated (Jaques *et al.* 1967, Güven and Ertan 1984, Güven and Güler 1989).

Heparin like substances have been isolated from marine animals such as *Stichopus japonicus* and *Halothuria leucophilota* (Holothuroidae, Actinopoda) (Burson *et al.* 1956, Ruan *et al.* 1986). Raparin was first obtained from *Rapana venosa* (Valenciennes) and was compared with heparin (Güven *et al.* 1991).

In this study, raparin was extracted from hepatopancreas part of *Rapana venosa* and its fractions were subjected to biological and analytical tests.

Materials

Rapana venosa (Valenciennes) was collected from the Turkish coasts of Black Sea.

Sephadex G-50 Superfine (Pharmacia),

Sheep plasma [Kraeber Co (Hamburg)],

Trypsin [Sigma (10 GT 8128)],

Heptest Kit (Haemachem, Inc.),

Hepaclot Kit (Diagnostica Stago),

Heparin (Sigma Grade I.,178 U/mg)

Azure A (Gurr, USA), Toluidine blue and all other chemicals are Merck products.

UV spectrophotometer (Shimadzu UV-VIS Recording, 160 A),

IR spectrophotometer (Shimadzu IR 435),

Freeze Dryer (Lyovac GT 2 Leybold, Heraeus),

Methods

1.Extraction techniques

Rapana venosa was dissected and the hepatopancreas part was separated (I) and raparin was obtained by using heparin extraction methods (Charles and Scott 1933, Kuizenga and Spaulding 1943).

a) Raparin RH_{500} *:* A sample (I) of 380 g was milled and macerated with ethyl alcohol for 24 h at $4^{o}C$ then filtrated. The residue was treated with 140 ml distilled water and 1 ml

xylene for 24 h at room temperature then added 400 ml 0.75 N sodium hydroxide solution and 50 ml saturated ammonium sulphate solution. The mixture was heated by stirring at 55°C in a water-bath for 2 h and at 60°C for 30 min. The macerate was filtred and kept at 80°C for 30 min and then acidified with sulfuric acid to pH 2.5. The crude raparin (RH_{500}) was separated by centrifugation and washed with ethyl alcohol five times and recentrifuged.

b) Raparin RHP_{10} : 150 g sample (**I**) was homogenized in a blender and macerated with petroleum ether for 24 h at 4°C, then was centrifuged and the residue was washed with ethyl alcohol for 24 h at 4°C and filtered. 0.75 N sodium hydroxide solution and saturated ammonium sulphate solution were added to the residue and heated while stirring at 55°C in a water-bath for 2 h and then at 60°C for 30 min. The macerate was filtered and kept at 80°C for 30 min and then acidified with sulfuric acid to pH 2.5. After centrifugation, the crude raparin (RHP_{10}) was washed with ethyl alcohol five times and recentrifuged.

c) Raparin RKL_{10}: 263 g sample (**I**) was homogenized, 97 ml distilled water and 1 ml xylene mixture were added and then heated by stirring at 35°C in a water-bath for 30 min. The mixture was macerated at room temperature for 24 h and 275 ml 0.75 N sodium hydroxide solution and 46 ml saturated ammonium sulphate solution were added. It was heated at 55°C in a water-bath for 2 h and at 80°C for 30 min and then was filtered. The filtrate was acidified with sulfuric acid to pH 2.5. After centrifugation, the residue was separated and washed with ethyl alcohol five times and recentrifuged. The residue was dissolved in 25 ml distilled water and pH value was brought to 8.5 with 2 N sodium hydroxide solution and 0.1 g trypsin was added. For tryptic fermentation, the mixture was maintained at 38°C for 60 h and precipitated with ethyl alcohol and then centrifuged. The crude raparin (RKL_{10}) was suspended in water and heated at 75°C in a water-bath for 40 min. and reprecipitated with acetone and centrifuged again.

2. Fractionation of raparin

The crude raparins were fractionated through Sephadex G-50 Super fine column. 35 g Sephadex G-50 Super fine was swollen in distilled water for 3 h and poured into a glass column (70x2.8 cm). The void volume was found to be 105 ml using dextran blue 2000.

200 mg crude raparin (RH_{500} / RHP_{10} / RKL_{10}) was suspended in 4 ml distilled water, centrifuged and filtered. The filtrate was applied to the Sephadex G-50 column and eluted with distilled water with a flow rate of 50 ml/h. 5 ml eluates were collected and its absorbances measured at 190 - 200 nm against distilled water. The elution curve for each sample was plotted and the fractions were seperated and lyophylised and their IR spectra were taken.

3. Coagulation time methods

3.1. Recalcification time (Foster and Nutley 1942)

500 · g/ml stock solutions of raparin fractions were prepared. 50, 100, 200 and 400 · g/ml stock solutions of raparin fraction were transfered to test tubes, priorly siliconized and 180 · l sheep plasma was added to each tube. The volume was adjusted to 1.0 ml with saline, then 200 · l 0.025 M calcium chloride was added and the coagulation time was determined. The control was made with 25 · l (0.25 U) heparin solution prepared from 10 U/ml Heparin Sigma G I stock solution.

3.2- Heptest and Hepaclot methods

The plasma used in this assay was obtained by mixing 9 parts of fresh human blood with one part of sodium citrate (3.8 %). Plasma was separated by centrifugation.

3.2.1. Heptest method (Yin *et al.* 1973)

Stock solutions of raparin and its fractions were prepared as 1 · g/ml. 25, 50 and 75 · l and put in test tubes at 37°C in a water-bath and added 25 · l human plasma and 0.1 ml Factor Xa (lyophilized and stabilized in a buffer containing serum albumin, PEG, NaCl, trismaleate at pH 7.5). After 120 sec., 0.1 ml Recalmix (lyophilized and buffered at pH 7.5) was added and the coagulation time was determined. The control was made by human plasma omitting raparin.

3.2.2. Hepaclot method (Kakkar 1974)

1 mg/ml stock solutions of raparin and its fractions were used. The test was performed at 37°C in a water-bath. 50 · l solution of AT III [purified bovine Antithrombin III (AT III), freeze-dried] and 25 · l solution of raparin were added to the tubes and incubated for 60 sec., then 0.1 ml Factor Xa (purified bovine Factor Xa, freeze-dried) added and incubated again for 90 sec. 0.1 ml substrate plasma (specially treated substrate plasma, freeze-dried) was added after incubation and 0.1 ml 0.025 M $CaCl_2$ was pipetted 30 seconds later the coagulation time was determined. The control was made by human plasma omitting raparin.

4. Metachromatic assay

The metachromasy with azure A and toluidine blue with raparin was tested.

0.2, 0.4 and 0.6 ml of the stock solutions of raparins (500-1000 · g/ml) were put into test tubes by microsyringe. The volume was adjusted to 1.1 ml with distilled water and 50 · l

barbitone buffer (1.105 g barbitone in 10 ml 0.5 M sodium hydroxide solution and 10 ml distilled water, then the volume adjusted to 100 ml with distilled water) and 50 · l dye solution (25 mg tested dye dissolved in 100 ml distilled water) were added. The maximum absorbance values of dye solutions and raparin+dye solutions were determined using UV spectrophotometer.

Results

1-The fractionation of raparin

Table 1 showed λ max. values of raparin and its fractions obtained from Sephadex column.

Table 1. λ max. of raparin and its fractions and elution volumes of fractions

Raparin	λ max. (nm)	Elution volumes (ml)
RH_{500}	196.1	-
RH_{500} Fr I	196.1	1-90
RH_{500} Fr II	198.5	205-300
RHP_{10}	193.9	-
RHP_{10}Fr I	193.7	1-60
RHP_{10}Fr II	191.1	170-240
RKL_{10}	198.5	-
RKL_{10}Fr I	204.9	1-100
RKL_{10}Fr II	194.3	160-280
Heparin Sigma GI	197.9	-

2-IR spectrums are shown for raparin RH_{500} and raparin RH_{500} F I in Figure 1,2 and for Heparin Sigma G 1 in Figure 3.

IR spectrum of RH_{500} and RH_{500} Fr I showed -SO3 band at 1220 cm^{-1} and 1020 cm^{-1}, COO^- band at 1620 cm^{-1}, 1371 cm^{-1} and 1021 cm^{-1}, -C-OS- band at 927 cm^{-1}, 847 cm^{-1} and also amid band at 1461 cm^{-1}.

Figure 1. IR spectrum of raparin RH_{500}

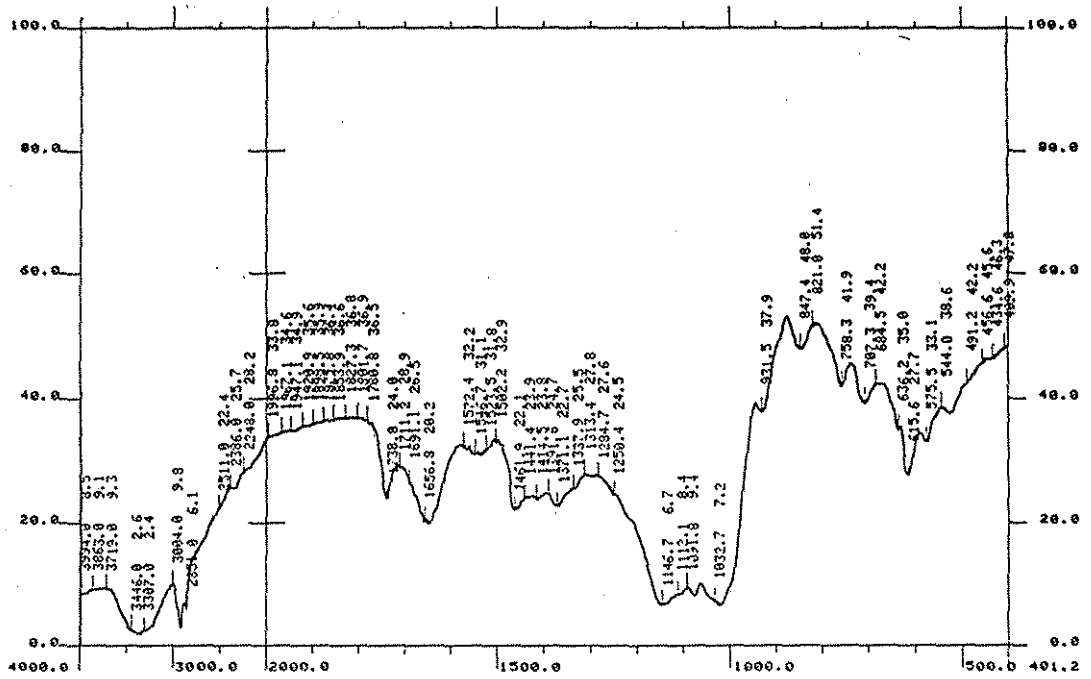


Figure 2. IR spectrum of raparin RH_{500} Fr I

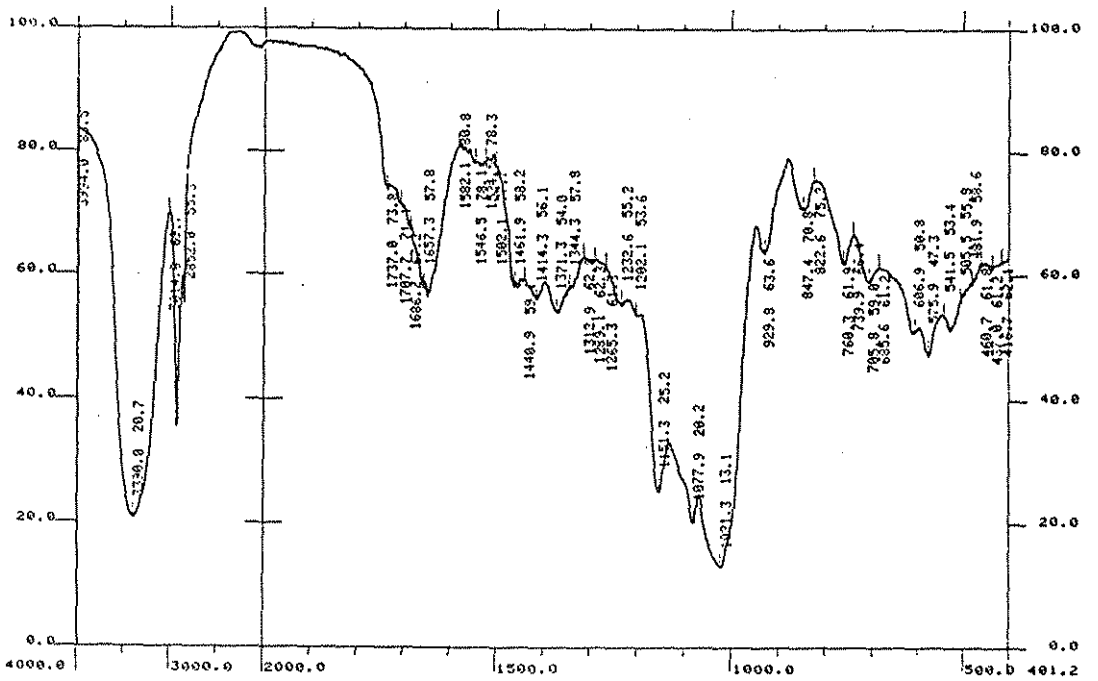
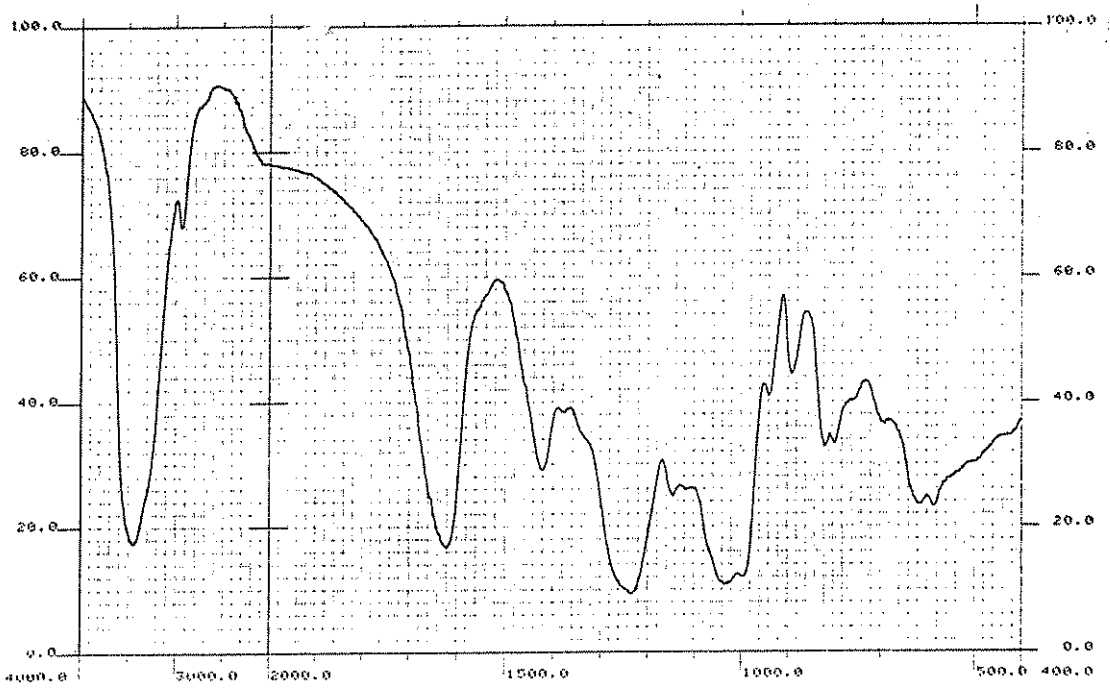


Figure 3. IR spectrum of Heparin Sigma G I



3. Coagulation time results

3.1. Recalcification time

The results of recalcification time of raparin fractions are shown in Table 2. Recalcification time of heparin is 385 sec and blank value is 198 sec.

Table 2. Recalcification time of different concentrations of raparin fractions

Fractions of Raparin	Recalcification time (sec.)			
	25 ' g	50 ' g	100 ' g	200 ' g
RH_{500} Fr I	433.0	401.0	322.4	268.2
RH_{500} Fr II	390.6	379.8	337.4	300.6
RHP_{10} Fr II	454.0	378.6	308.2	237.8
RKL_{10} Fr I	474.4	308.6	310.2	189.8
RKL_{10} Fr II	470.2	393.3	291.4	207.0

All fractions of raparin showed anticoagulant activity. The highest activity was observed with RKL_{10} Fr I, RH_{500} Fr I, RH_{500} Fr II and RH_{500} Fr II.

3.2. Heptest and Hepaclot methods results

The results of Heptest and Hepaclot methods are shown in Table 3.

In Heptest results, the highest activity was found with raparin RHP_{10} fraction. The activities of the other fractions were ranked: RHP_{10} Fr II > RKL_{10} > RH_{500} > RKL_{10} Fr II > RH_{500} Fr I > RH_{500} Fr II > RKL_{10} Fr I.

In Hepaclot results the highest activity was found with RHP_{10} Fr II. The activities of the other fractions were ranked. RKL_{10} Fr II > RKL_{10} Fr I > RH_{500} > RH_{500} Fr I.

Table 3. The results of Heptest and Hepaclot (sec)

Conc. / Raparin and its fractions	Methods		
	Heptest*		Hepaclot**
	25 ' g	75 ' g	25 ' g
RH_{500}	20.0	40.0	15.0
RH_{500} Fr I	19.8	39.0	14.2
RH_{500} Fr II	21.0	38.2	13.0
RHP_{10}	23.0	84.0	14.0
RHP_{10} Fr II	21.0	48.0	20.2
RKL_{10}	19.6	43.0	14.0
RKL_{10} Fr I	19.2	33.8	15.0
RKL_{10} Fr II	19.0	39.8	15.2

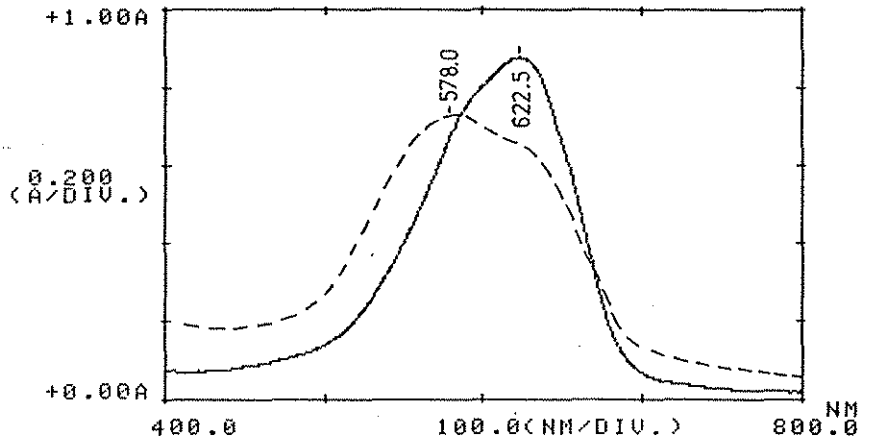
*Control : 20 sec

** Control : 13.8 sec.

3.3.Metachromasy with azure A and toluidine blue

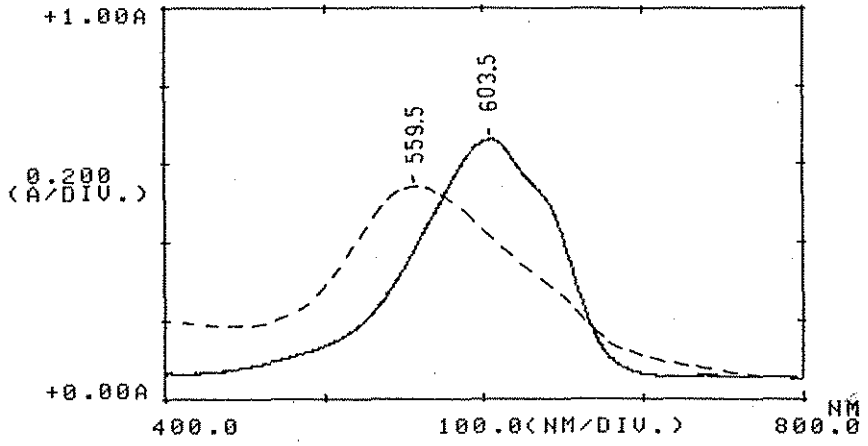
RH_{500} Fr I gave metachromasy with tested dyes. The absorption spectra of dyes and raparin RH_{500} Fr I + dyes are shown in Figure 4 and 5.

Figure 4. The absorption spectrum azure A (——) and azure A + raparin RH_{500} Fr I (----)



λ max. value of dye decreased with tested fraction from 622.5 to 578 nm.

Figure 5. The absorption spectrum toluidine blue (——) and toluidine blue + raparin RH_{500} Fr I (---)



λ max. value of dye decreased with tested fraction from 603.5 to 559.5 nm.

Discussion

Raparin was isolated from *Rapana venosa* in 1991. In this study, raparin was extracted by three different techniques and was fractionated through Sephadex G-50 Superfine column and two fractions were obtained. Raparin and its fractions were tested by biological methods as recalcification time, Heptest, Hepaclot tests. All fractions showed activity in recalcification time assay and RKL_{10} Fr I showed the highest activity. According to Heptest results all fraction showed activity and RHP_{10}(crude heparin) showed hhe highest activity. Only RHP_{10} Fr II showed considerable activity with Hepaclot test. RHP_{10} Fr II showed similar results in Heptest and Hepaclot to that of High Molecular Weight Heparin (HMWH). The reason for the inactivity of RKL_{10}(crude heparin) in Hepaclot method although it showed the highest activity in Heptest method can be explained by the fact that this fraction is similar to Low Molecular Weight Heparin (LMWH) in effect. The other fractions gave negligeable activity in both tests. Only fraction I of raparin RH_{500} showed metachromatic activity with the dyes azure A and toluidine blue.

IR spectrums of raparin and its fractions were compared to heparin Sigma G I. The S = O bands at 1225 cm^{-1} and 1040 cm^{-1} and -C-OS- band at 820 cm^{-1} of raparin are similar to heparin. In general the bands of RH_{500} and RH_{500} Fr I are similar to heparin spectra. The bands 1738 cm^{-1} and 1656 cm^{-1} differ from that of heparin.

Özet

Heparin ilk olarak 1916'da köpek karaciğerinden izole edilmiştir. Yap lan son çal şmalarda, deniz hayvanlar ndan heparin benzeri madde izole edilmiştir. Yeni olarak *Rapana venosa*'dan Raparin ad verilen heparine benzer madde 1991'de izole edilmiştir. Bu çal şmada, üç farkl teknikle *Rapana venosa*'n n hepatopakreas ndan Raparin elde edilmiştir ve bunlar Sephadex G-50 Super fine kolondan geçirilerek fraksiyonland r lm ş ve herbirinden iki fraksiyon elde edilmiştir. Bu fraksiyonlarda, p ht laşma zaman , Heptest, Hepaclot testleri ve metakromatik aktivite çal şmalar yap lm şt r. Bütün fraksiyonlar p ht laşma zaman tayininde aktivite göstermiş ve bu tayinde Raparin RKL_{10} Fr I'in en yüksek aktivitede olduğu saptanm şt r. Heptest sonuçlar na göre başta RHP_{10} olmak üzere bütün fraksiyonlar aktivite göstermiş, Hepaclot testinde ise en yüksek aktiviteyi Raparin RHP_{10} Fr II'nin gösterdiği saptanm şt r. RHP_{10}'un sadece Heptest metodunda aktivite göstermesi nedeniyle düşük molekül ağ rl kl heparine, RHP_{10} Fr II'nin ise her iki testte de aktivite göstermesi ile yüksek molekül ağ rl kl heparine benzer etkide olduğu ve RKL_{10}'un aktivite yönünden RHP_{10}'a benzediği yorumlanm şt r. Elde edilen fraksiyonlardan sadece Raparin RH_{500} Fr I azure A ve toluidine blue boyalar ile metakromatik aktivite göstermiştir. Raparin ve fraksiyonlar n n IR spektrumlar heparin ile mukayese edilmiş ve RH_{500} ile RH_{500} Fr I'e ait spektrumun heparine benzer olduğu saptanm şt r.

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