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INHIBITORY EFFECT OF FLAVONOIDS ISOLATED FROM SOME *JUNCUS* SPECIES (FLOWERS & CALLUS) ON BLOOD PLATELETS AGGREGATION

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تم دراسة تأثير الجرعات المختلفة من الخلاصات المحضرة من نبات سمار المر وحصر الجين والأنسجة المنزرعة لنبات سمار المر وكذلك المواد النقية المنفصلة على الصفائح الدموية للإنسان. ولقد لوحظ أن مجموعات الهيدروكسيد الموجودة فى المركبات لها تأثير كبير على فاعلية المركبز فزيادة عدد مجموعات الهيدروكسيد فى المادة تقلل من فاعليتها على الصفائح الدموية. كما تم تعيين كميات الفلافونيدات المفصولة وذلك باستخدام التحليل الكروماتوجرافى والكيميائى وطرق التحليل الألى.

The anti-platelets aggregation effect of the different dose levels of plant extracts prepared from the flowers and the tissue culture as well as the pure isolated flavonids were tested using human blood of healthy fasting volunteers. The hydroxylation pattern of the tested compounds affecting their activities. The more number of (OH) group the lesser activity of the compound. The isolated flavonoids were quantitively determined by chromatographic & spectrophotometric methods of analysis.

INTRODUCTION

Family Juncaceae is a wide spread family. Its plants are growing wild in Egypt especially in damp habitate, salty lands and sandy localities.

In case of tissue culture, the medium composition is highly affecting the callus formation and its active constituents.[1]

J. species are rich in flavonoids which have several activities they were anciently used as diuretic and for urinary troubles recently they have anti-plateletes aggregation anti-allergic, anti-inflammatory, anti-viral and anti-carcinogenic effects.[2-5]

In-vivo platelets participate in primary hemostasis by first adhering and then aggregating at the site of an injured blood vessel, platelets aggregation can be followed *in-vitro* by adding inducers such as collagen to stirred platelets rich plasma (PRP).

The increase in light transmittance is recorded as the platelets aggregate, the absorbance (OD) change is measured and recorded as the platelets aggregate (Triplett).[6]

MATERIALS AND METHODS

The flowering tops of *Juncus acutus* L. and *J. rigidus* C.A. Mey were air dried and pulverized. Tissue culture prepared from the seeds of the above mentioned plants. Culture media as 4X, White and Murashige and Shoog's.[7-9] Growth regulators: 0.01% solution, as IAA, 2, 4 dichlorophenoxy acetic acid & kinetin. Collagen (Helena platelets aggregaton reagents laboratories products). Citrate phosphate dextrose solution as anti-coagulant.

The third generation callus was formed on 4X solid media from the sterile seeds according to street, H. E. procedure.[10] The ingrediants of each midium of the two forms (liquid & solid), were throughly mixed. Twenty flasks for each of two forms of the three media were prepared, about half gram of callus from the third generation, was transferred asceptically to each flask and incubated in a shaker incubator with agitaton rate from 100-150 rpm with motion stroke of 3-4 cm. at 20-25° for 6-8 weeks.

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Various culture parameters viz age, pH and hormonal concentration were studied on 4X solid medium to show their effect on the formed callus for both species. The yield of fresh and dry weights were used as a factor to choose the suitable state for the above parameters. The results are illustrated in Tables (1-7).

The flavonoids of *Juncus acutus* L. and *Juncus rigidus* C. A. Mey flowering tops were isolated according to the procedure mention in litrature number. [11]

To isolate and charactrize flovonoidal components of both *Juncus* species, five grams from each of the air dried defatted powdered flowering tops of *J. rigidus* C. A. Mey., *J acutus* L. and the tissue culture of *J. acutus* L. were successively exhausted with ether and methanol. The solvents were distilled off and the residues were separately dissolved in 10 ml methanol. The obtained solutions from ether residues were kept for the chromatographic separation of aglycones and that obtained from methanol residues were kept for chromatographic separation of glucosides.

The maximum absorbance of each of the authentic flavonoids was determined by dissolving an aliquot equivalent to 1 mg of each of luteolin, luteolin-7-glucoside, apigenin, apigenin-7-glucoside and hesperetin in 100 ml methanol. The results are represented in Table (8).

Several aliquots of the flavonoids under tests equivalent to 2, 4, 6, 8 and 10 μg/ml were measured at the specific λ max for each, the results are summarized in Table (9).

The percentage recovery for each flovonoids under test was determined by applying one millilitre of 0.003% methanolic solution on Whatman No. 3 paper chromatography for glucoside, developed with solvent system acetic acid: water (15:85). While for aglycone silica gel G plates and solvent system chloroform-methanol (90:10) were used.

The percent recovery, E, was found to be 80% for luteolin-7-glucoside, 85% for apigenin-7-glucoside, 92, 93 and 94 for luteolin, hesperetin and apigenin respectively.

The same procedure was applied for quantitative separation of the individual component in the extracts.

Results are represented in Table (10)

The anti-platelets aggregation effect of the isolated flavonoids was determined adopting Alain-B. *et al.* procedure[6]:

1- Blood samples 9 ml each, were taken from a forearm vein of human healthy fasting volunteers who had not adminsatered any drugs with special stress to aspirin or aspirin containing drugs for at leat 7-10 days period to the expermints, then added to 1 ml of citrate phosphate dextrose solution, as anticoagulant, in plastic test tube. Platelets rich plasma (PRP) was obtained from the supernatant of centrifuged citrated blood, at speed of 1000 rpm for 10-15 minutes at room temperature. Platelets poor plasma (PPP) was obtained from the supernatant of recentrifugating remaining blood samples at 3000 rpm for 10-15 minutes at room temperature.
2- Prepare the aggregation recorder for use.
3- 0.45 ml from each of platelets rich plasma (PRP) and platelets poor plasma (PPP) were separately pipetted into different cuvette and incubated for 5 minutes at 37°.
4- The PPP specimen was inserted into the appropriate Channel and instrument was set to 100% aggregation then remove the specimen.
5- A stir bar was added to PRP specimen and the tube was inserted into the recorder.
6- 0.05 ml of the collagen "aggregation agent" brought to room temperatureat 15-30° before use was added to the PRP and record the aggregation.

To choose the suitable concentration of the drugs under test which gave the resonable effect when challenged with collagen an aliquots equivalent to 10, 20, 30 μl of 0.5 mg/ml solutions of each of the drug extracts under investigation as well as the pure isolated compounds luteolin, apigenin and hesperatin were placed in several cuvettes containing (PRP), then collagen was added. The percentage of aggregation was recorded automatically on the instrument and the results are represented in Figures (1-3) and Table (11).

Table 1: The compostition of the three differant media.

Composition	Concentration of the components in each medium (mg/L)		
	4X[108]	White[109]	Murashige & shoog[100]
Macroelements			
$MgSO_4.7H_2O$	250	720	370
Na_2SO_4	--	200	--
KCl	--	65	--
$CaCl_2.2H_2O$	150	--	440
KNO_3	2500	80	1900
$Ca(NO_3)_2.4H_2O$	--	300	--
NH_4NO_3	--	--	1650
$NaH_2PO_4.H_2O$	150	16.5	--
KH_2PO_4	--	--	170
$(NH_4)_2SO_4$	134	--	--
Microelements			
KI	0.75	0.75	0.83
$MnSO_4$-H_2O	10.0	7.0	22.3
H_3BO_3	3.0	1.5	6.2
$ZnSO_4.7H_2O$	3.0	3.0	8.6
$CuSO_4.5H_2O$	0.025	--	0.025
$NaMoO_4.2HO$	0.025	--	0.025
$CoCl_2$	0.025	--	--
$FeSO_4.7H_2O$	13.9	2.5	27.85
$AlCl_3$	--	--	0.025
	4X[109]	White[110]	Murashige & shoog[101]
Additives			
Na_2 EDTA	18.6	--	37.25
Casein hydrolysate	2500	--	--
Sucrose	20000	20000	30000
Ca-pantothenate	--	1.0	--
Agar	8000	8000	8000
Vitamins			
Thiamine-HCl	10.0	0.1	0.1
Pyridoxine-HCl	1.0	0.1	0.5
Glycine	--	3.0	2.0
Nicotinic acid	1.0	0.5	0.5
Meso-inositol	100	--	100
Cystenic HCl	--	1.0	--
Growth regulators			
Kinetin	2.0	2.0	2.0
Indole acetic acid (IAA)	0.5	0.5	0.5
2,4-Dichloro phenoxy acetic acid (2,4D)	2.0	2.0	2.0

Table 2: The effect of different media on the callus growth of both *Juncus* species.

Media name	*Callus weight /gm for							
	Juncus acutus L.				*Juncus rigidus* C.A. Mey			
	Solid medium		Liquid medium		Solid medium		Liquid medium	
	Fresh	Dry	Fresh	Dry	Fresh	Dry	Fresh	Dry
4X	13.1	0.54	14.3	0.56	12.5	0.49	13.6	0.55
White's	10.3	0.38	11.0	0.45	9.0	0.36	9.3	0.37
Murashige and Shoog	12.1	0.49	12.5	0.50	10.5	0.40	10.6	0.40

*Average of three determinations.

Table 3: The effect of age on callus fresh and dry weight for both *Juncus* species.

Age / day	Callus Weights / gm for			
	J. acutus L.		*J. rigidus* C.A. Mey	
	Fresh	Dry	Fresh	Dry
5	0.85	0.014	0.7	0.008
10	1.50	0.050	1.2	0.040
15	2.20	0.080	2.1	0.080
20	5.10	0.200	5.0	0.200
25	8.70	0.360	9.1	0.370
30	11.30	0.460	9.7	0.390
35	12.70	0.540	10.2	0.400
40	12.10	0.510	10.9	0.450
45	11.50	0.470	10.7	0.430
50	11.00	0.450	10.2	0.400

Table 4: The effect of pH on the fresh and dry weights of the callus for both *Juncus* species.

pH	*Callus Weight /gm for			
	J. acutus		*J. rigidus* A.C. Mey	
	Fresh	Dry	Fresh	Dry
4	8.3	0.33	8.1	0.32
4.5	10.1	0.40	9.7	0.39
5.0	12.2	0.51	10.5	0.43
5.5	12.8	0.53	11.2	0.46
6.0	12.0	0.48	10.3	0.41
6.5	10.1	0.40	9.1	0.37
7.0	8.5	0.34	8.0	0.32

*Average of three determinations.

Table 5: The effect of kinetin on the callus fresh and dry weights of both *Juncus* species.

Conc. of kinetin /mg	*Callus Weight /gm for			
	J. acutus		*J. rigidus* A.C. Mey	
	Fresh	Dry	Fresh	Dry
Zero	12.50	0.550	11.0	0.46
1	12.10	0.510	10.6	0.43
2	11.50	0.480	9.1	0.37
3	9.50	0.380	8.5	0.35
4	7.10	0.290	7.0	0.29
5	4.60	0.170	4.1	0.14
6	0.83	0.013	0.9	0.02
7	0.50	0.010	0.5	0.01

*Average of three determinations.

Table 6: The effect of indol-3-acetic acid on the callus fresh and dry weight for both *Juncus* species.

Conc. of IAA/mg	*Callus Weight /gm for			
	J. acutus		*J. rigidus* A.C. Mey	
	Fresh	Dry	Fresh	Dry
Zero (Blank)	3.4	0.12	3.0	0.11
0.2	6.9	0.29	4.3	0.15
0.4	11.0	0.45	9.2	0.38
0.5	12.5	0.53	10.0	0.40
0.6	12.7	0.55	11.4	0.47
0.8	13.5	0.60	11.6	0.49
1.0	12.7	0.55	10.3	0.41
1.2	9.9	0.40	9.1	0.37

*Average of three determinations.

Table 7: The effect of 2,4D on the callus fresh and dry weights for both *Juncus* species.

Conc. of 2,4 D/mg.	*Callus Weights /mg for			
	J. acutus L.		*J. rigidus* C.A. Mey	
	Fresh	Dry	Fresh	Dry
Zero (Blank)	4.3	0.15	5.2	0.20
1	11.2	0.46	10.4	0.42
2	13.1	0.57	11.5	0.47
3	11.8	0.49	10.7	0.43
4	9.0	0.37	8.3	0.34
5	7.5	0.32	7.8	0.32

Table 8: The maximum absorbance of luteolin, luteolin-7-glucoside, apigenin, apigenin-7-glucoside and hesperetin in methanol.

Flavonoids	Luteolin	Luteolin-7-glucoside	Apigenin	Apigenin-7-glucoside	Hesperetin
λ max. at	345	345	335	335	287

Table 9: Flavonoids absorbance at different concentrations.

Conc. µg/ml	Absorbance				
	Luteolin	Luteolin-7-glucoside	Apigenin	Apigenin-7-glucoside	Hesperetin
2	0.135	0.082	0.169	0.090	0.135
4	0.265	0.183	0.326	0.173	0.270
6	0.400	0.265	0.490	0.260	0.404
8	0.469	0.356	0.656	0.346	0.538
10	0.634	0.456	0.826	0.432	0.673

Table 10: The percentage of the isolated flavonoids from both *Juncus* species flowers and *J. acutus* L. formed callus.

Isolated flavonoids	Percentage gm w/w		
	J. rigidus C.A. Mey	*J. acutus* L.	
	Flowers	Flowers	Tissue culture
1) Luteolin-7-glucoside	4.910	4.800	0.190
2) 7-methoxy-luteolin-4'-glucoside calculated as luteolin-7-glucoside	2.100	1.900	--
3) Apigenin-7-glucoside	1.100	0.700	1.060
4) 7-methoxy-apigenin-4'-glucoside calculated as apigenin-7-glucoside	0.400	0.600	0.910
5) Luteolin	0.550	0.390	0.161
6) 7-methoxy-luteolin calculated as luteolin	0.300	0.120	--
7) Apigenin	0.035	0.030	1.020
8) 7-methoxy apigenin calculated as apigenin	0.015	0.022	0.500
9) Hesperetin	--	--	0.085

Table 11: Percentage of platelets aggregation of pure flavonoids at different doses level.

Dose µl	Luteolin x factor 1.05	A pigenin	Hesperetin	Ethyl alcohol extract	Ether extract
Control	99.0	99.5	99.5	Control 80	90
10	66.9	74.5	77.3	J.A. 53	40
20	42.1	40.9	53.2	J.R. 50	40
30	43.5	41.4	41.4	T.C. 52	12

Table 12: Persentage of platelets aggregation of extracts of *J. acutus*, *J. rigidus* and formed callus.

Plate extract	Ethyl acetate extract	Ether extract
Control	80	90
J. A.	53	40
J. R.	50	40
T. C.	52	12

RESULTS AND CONCLUSION

As shown in Table (1) the suitable medium for the growth of both *Juncus* species was the two forms of 4X medium. This result may be attributed to the moderate concentration of the elements in this medium in relation to the other tested media, where White's medium contains low salts contents and Murashige's, Shoog medium contains high salts contents as previously shown in Table (2).

In comparing the two form of 4X medium it was clear that 4X liquid medium gave lore and rapid callus formation than the solid form. This result referred to the agitation used in this case which leads to the homogenity of the content inside the medium during the callus growth in addition it gave a damp, salty land for the formed callus.

The 4X liquid medium has the same character of the land used for the parent plant, but it was easily contaminated, so that the collected callus from 4X solid medium were used as stock callus for further studies.

Tables (3-4) showed that both fresh and dry weights of the callus increased progressively up to the age 35 day for J.A.L. and 40 day for J.R.C.A. May at pH 5.5 and then decrease with further increase in age and pH.

Table (5) showed that maximum callus growth of both *Juncus* species was performed in the absence of kinetin "i.e" blank with zero concentration, the callus growth was decreased with the increase of kinetin concentration. This decrease was noticed as slow rate from zero till 2 mg concentration of kinetin (the normal concentration in 4X medium.[7] After that the rate was decrease with steady rate till reached 5 mg/L of kinetin then followed by a sharp decrease in the formed callus ar both 6, 7 mg/L concentration. Generally, it was reported that monocotyledons do not need any cytokinin during the callus growth due to their high content of endogenic factor.[12]

Also the high concentration of kinetin has toxic effect on the formed callus. Our results were in agreement with those showed by Mitsugu *et al.* (1979)[12] on rice culture and other cereals cultures.

Table (6) showed that the callus growth in both *Juncus* species was gradually increased with the increase of IAA concentration. All the tested doses of IAA showed higher results than those of the blank zero concentration. The maximum yield of callus was observed at 0.8 mg/L concentration of IAA. The normal concentration of IAA in the normal 4X medium was 5 mg/L which showed higher increase in the callus growth but it was lower than that obtained with 0.8 mg concentration of our expermental results.

IAA is a natural auxin produced inside the plant cells during its growth. IAA has a positive effect on the growth of the cells, in our experment it induced callus formation till 0.8 mg concentration and after that it becomes inactive or its effect becomes lower.

Table (7) showed that the callus growth of *J. acutus* L. and *J. rigidus* C.A. Mey increased gradually with the increase of 2,4D concentration. All the tested doses showed higher values than those of the blank (zero concentration). The maximum yield was observed at 2 mg/L concentration which is the normal concentration of 2,4D in the 4X medium. After that concentration the callus growth was decreased gradually. It was reported that the cell and tissue cultures of monocotyledons require as a rule high concentrations of Auxins.[13-16]

So the combination of natural and synthetic Auxin have a potential effect on the callus growth but with specified concentrations.

From Table (10) one can conclude the following:

1- Both *Juncus* species flowering tops, were rich in flavonoid glucoside content.
2- *Juncus rigidus* C.A. Mey flowers showed higher result in comparing with the *Juncus acutus* L. flowers.
3- As regard to the indivedual components luteolin 7-glucoside was the major glucoside in both *Juncus* species flowers. It was detected in higher percentage in case of *Juncus rigidus* C.A. Mey flowers followed by a decreasing order with the flowers of *J. acutus* L.
4- Lutiolin-7-methoxy-4'-glucoside was the second glucoside detected in both species it was, present with some what higher percentage in *J. rigidus* C.A. Mey flowers then the *J. acutus* L. flowers but the tissue culture of the latter species was totaly free from this glucoside.
5- The pattern was inverted for the total aglycone content. *Juncus acutus* L. tissue culture was superior in this respect when compared with the flowering tops of both *Juncus* species.
6- The detected aglycones in the studied organ in both species were luteolin, 7-methoxy, luteolin, apigenin and 7-methoxy apigenin. The same aglycones, with the exception of 7-methoxy luteolin were detected in *J. acutus* L. tissue culture in addition to hesperetin which was not detected in both Juncus flowers.
7- Luteolin was detected in higher percentage in *J. rigidus* C.A. Mey flowers followed by a decreasing order with *J. acutus* L. flowers then with *J. acutus* L. tissue culture. Apigenin was present in higher percentage in tissue culture, it also considered as the major flavonoid constituents of the tissue culture.

From Figures (1, 2) one can conclude the following:

1- The ether extracts of both *Juncus* species flowers and that of *J. acutus* L. tissue culture have antiaggregating power. Tissue culture have more signifficant effect than the other two extracts, this result may be attributed to the presence of high concentration of apigenin in addition to the presence of hesperetin in that tissue. The hydroxylation pattern of the tested compounds affecting their activites. The more number of (OH) group the lesser activity of the compound. Also the substituted compound, methoxylated form have higher activity than the non methoxylated one.

   Accordingly the extracts which contain apigenin and methoxy apigenin had powerful effect than the those containing luteolin and methoxy luteolin. The result obtained by the ether extract of both *Juncus* species flowers which contain high concentration of luteolin and 7-methoxy luteolin had less antiaggregation power than the *J. acutus* L. tissue culture ether extract which was rich in apigenin and 7-methoxy apigenin.
2- The ethyl acetate extracts of the tested organs of both plants had nearly the same antiaggregation power but these effects were much less than that obtained by the ether extracts. These results were attributed to the presence of high polar compound flavonoid glucosides which have no penetration power to the plasma membrane in ethyl acetate extracts.
3- As regard to the pure isolated components Table (11) & Figures (3-5) especially luteolin and apigenin at the tested doses 10, 20 µl gave a significant antiaggregating power. 20 µl doses of both aglycones showed higher depression in platelets aggregation in comparing with 10 µl doses of the same compound.

   The depression that obtained by apigenin at 20 µl dose was higher than that obtained by luteolin at the same dose. The increase in the dose higher than 20 µl showed no effect on the platelets aggregation, so 20 µl dose was the critical dose.
4- In case of hesperetin the increase in the dose usually accumband with an increase in the depression of platelets aggregation, 30 µl dose of hesperetin had similar action to 20 µl dose of apigenin i.e. hesperetin had lower effect than apigenin at one and the same dose. These results may be attributed to the absence of double bond at C_2 in the righ C of hesperetin.

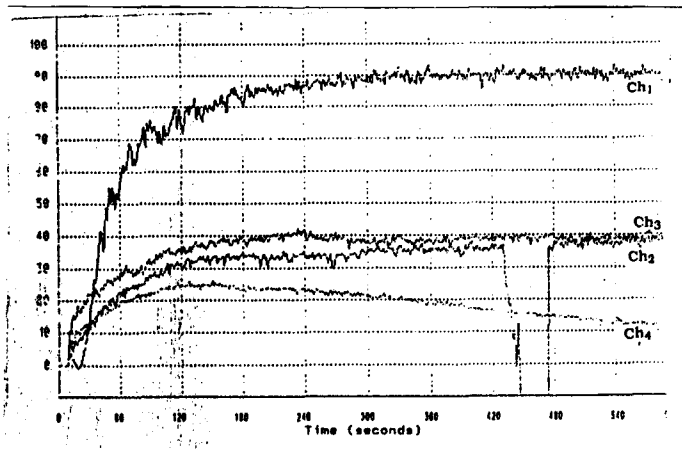

Fig. 1: Platelets aggregation assay for both *Juncus species* ether extracts and the formed *J. acutus* L. callus.

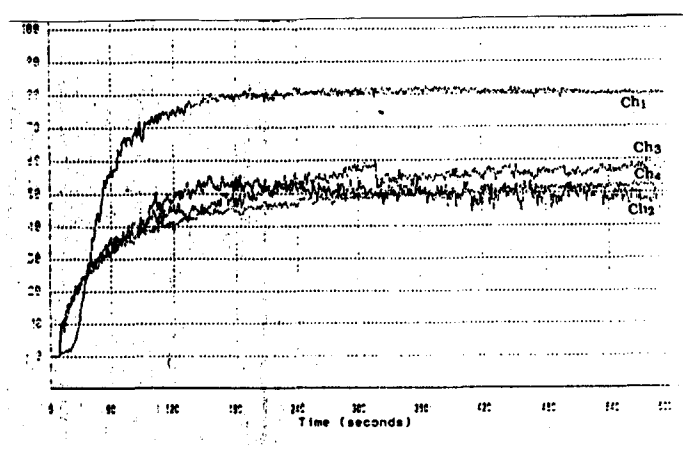

Fig. 2: Platelets aggregation assay for both *Juncus* species ethyl acetate extracts and the formed *J. acutus* L. callus.

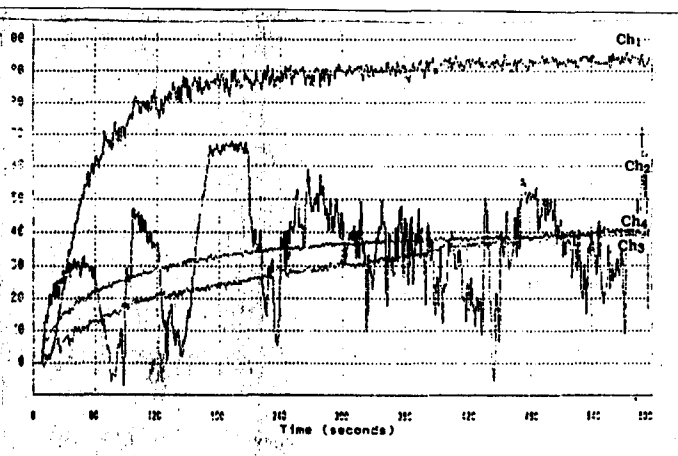

Fig. 3: Platlets aggregation assay of luteolin at differnet dose level.

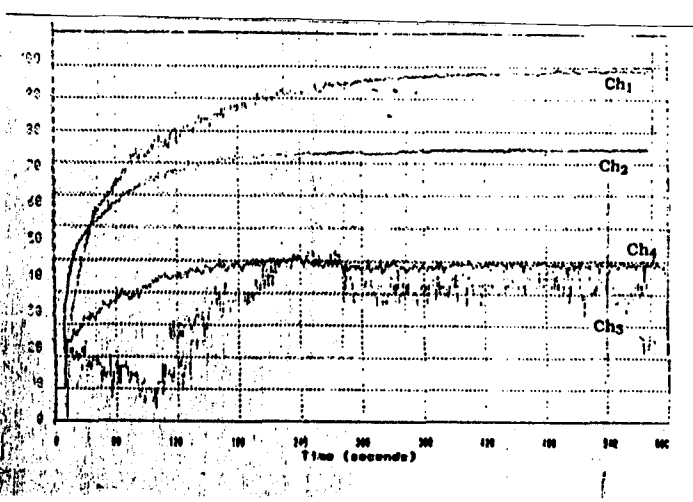

Fig. 4: Platelets aggregation assay of apigenin at differnet dose level.

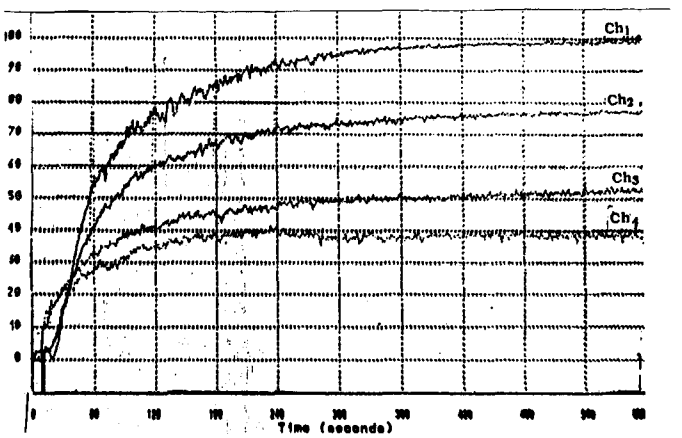

Fig. 5: Platelets aggregation assay of hesperetin at different dose level.

REFERENCES

1- A. Stefani, I. Arduni and A. Onnis, Annales Botanici. Fennici., 28 (1), 37 (1991).

2- B. Havsteen, Biochem. Pharmacol., 32, 1141 (1984).

3- L. Farkas, M. Gabor and F. Kallay, In "Flavonoids and Bioflavonoids", Akademiai Kiado, Budapest (1985).

4- H. Lshitsuka, X. Ohsawa, T. Ohiwa, I. Umeda and Y. Suhara, Antimicrob. Agents Chemother., 22, 611 (1982).

5- N. P. Das, "Flavonoids in Biology and Medicine", III National University of Singapore, Singapore (1989).

6- B. Alain, C. Jean-Peirre and A. Robert, Agents and Actions, 12, 3 (1982).

7- H. J. Bauch and E. Leistner, Planta Medica, 33, 150 (1978).

8- P. R. White, "The Cultivation of Animal and Plant Cells", New York, Ronald press (1963).

9- R. A. Dixon, "Plant Cell Culture a Practical Approach", Royal Holloway Collage Oxford England, 14 (1985).

10- H. E. Street, "Plant Tissue and Cell Culture", Botanical Monographs, 1st ed., University of California Press Berkeley, 11 (1973).

11- A. H. H. Hashem, A.A. Ahmed and S. G. Camelia, "A Pharmacognostical Study *Juncus acutus* L. Growing in Egypt", Ms.C. Thesis, Faculty of Pharmacy, Assiut University (1981).

12- I. Mitsugu, "Plant and Cell Physiol', 20 (5), 917 (1979).

13- A. Cattoir-Reynaeerts, M. Jacobs and G. Barley, Newsletter, 8, 23 (1978).

14- D. S. Brar, Z. Pflanzenphysiol 95 (5), 377 (1979).

15- H. W. Rines and T. J. McCoy, Crop Sci., 21 (6), 837 (1981).

16- B. V. Conger, Environ. and Exp Bot., 22 (1), 39 (1982).