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RANA RİDİBUNDA DERİ SEKRESYONU : ANTİFUNGAL ETKİSİ, KÜLTÜRDE YETİŞTİRİLEN RATTUS NORVEGİCUS EMBRYO FİBROBLAST VE KİMYASAL OLUŞTURULMUŞ TÜMOR HÜCRELERİNİN DNA SENTEZİNE VE MORFOLOJİSİNE ETKİSİ VE SAFLAŞTIRILMASI

THE RANA RIDIBUNDA SKIN SECRETION : ITS ANTIFUNGAL EFFECT, ITS EFFECT ON MORPHOLOGY AND ON DNA SYNTHESIS OF THE NORMAL EMBRYONIC FIBROBLAST CELLS AND CHEMICALLY INDUCED TUMOR CELLS OF RATTUS NORVEGICUS IN CULTURE AND ITS PURIFICATION

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SUMMARY

The extract (SSE) obtained from the skin secretion of the water frog Rana ridibunda, was purified by 95% cut $(NH_4)_2SO_4$ precipitation, Sephadax G25 or G75 and DEAE Sephadex A25 column chromatographies. All of the biological activities were peresent in the II. protein fraction of Sephadex G75 column chromatography. Antibacterial, antifungal, DNA synthesis inhibitor activities were present in the I. protein fraction of DEAE Sephadex A25 column chromatography whereas the II. protein fraction had glycoprotein structure with fibrinolytic system inhibitor activity.

Besides the antibacterial activitiy of the extract, the antifungal activity against 11 fungi strains (Candida albicans 7650, C. parapsilosis KUEN 1010 (Y) C_1–12–1, C. krusei KUEN 1001 (Y) C_1–6–3, C. albicans klaur A–2, C. albicans, C.tropicalis KUEN 1022 (Y) C_1–19–3, C. stellatoidea KUEN 1018 (Y) C_1–18–1, C. albicans MIV–211, Cryptococcus neoformans KUEN 1047 (Y) C_2–1–9, Rhodotorula glutinis KUEN 1064 (Y) R_1–1–1, Saccharomycea cerevisiae, dermatofites Microsporum nanum KUEN 1089 (F) M_5–6–1, Trichophyton mentagrophytes 703.) was also detected. This extract was ineffective against Torulopsis glabrata. A short review of this topic is also included.

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The effect of SSE on ^{3}H–thymidine incorporation into Rattus norvegicus embryonic fibroblast cells (REF) and tumor cells (RT) in tissue culture was followed by autoradiography and morphological alterations caused by SSE were followed by light microscopy.

In tissue culture, in the medium with 5% SSE after 17 hrs of incubation, morphological alterations in REF and RT were observed. After 39 hrs of incubation at 10% SSE, the DNA synthesis was inhibited in both REF and RT. In 5% SSE containing medium after 39 hrs of incubation, ^{3}H–thymidine incorporation into RT was 9.02 % whereas in control group (without SSE) this ratio was 36.62 %. The ^{3}H–thymidine incorporation into REF after 39 hrs of incubation in a medium with 5 % SSE was 6.05 % whereas this ratio was 40.10 % in the REF cells grown in a medium without SSE. The DNA synthesis inhibitor effect of SSE was abolished when the cells, both REF and RT, were transferred into a medium without SSE. The time of recovery was found to be directly proportional to the time of preincubation of the cells in the medium with SSE.

ÖZET

Su kurbağası Rana ridibunda'nın derisinden salgılanan ekstre (KDS), % 95 $(NH_4)_2SO_4$ presipitasyonu, Sephadex G25 veya G75 ve DEAE, Sephadex A 25 kolon kromatografileri sonucu saflaştırılmıştır. Sephadex G75 den elde edilen 2 protein fraksiyonundan birinde tüm biyolojik aktiviteler saptanmıştır. DEAE Sephadex A25 kolon kromatografisinden elde edilen I. protein fraksiyonundan antibakteriyel (AA) antifungal (AFA) ve DNA sentezi inhibisyon (DNA–SIA) aktiviteleri görülürken II. protein de glikoprotein yapısı ve fibrinolotik sistemi inhibe edici aktivite saptanmıştır.

Ekstrenin antibakteriyal etkisi yanında 11 maya suşuna (Candida albicans 7650, C. parapsilosis KUEN 1010 (Y) C_1–12–1, C. krusei KUEN 1001 (Y) C_1–6–3, C. albicans klaur A–2, C. tropicalis KUEN 1022 (Y) 1–19–3, C. Stellatoidea KUEN 1018 (Y) C_1–18–13, C. albicans MIV–211, Cryptococcus neoformans KUEN 1047 (Y) C_2–1–9, Rhodotorula glutinis KUEN 1064 (Y) R_1–1–1, Saccharomycea cerevisiae, dermatofitlerden Microsporum nanum KUEN 1089 (F) M_5–6–1, Trichophyton mentagrophytes 703) antifungal etkisi saptanmıştır. Torulopsis glabrata'ya etkili bulunmamıştır.

Rattus norvegicus embriyo fibroblast hücrelerine ve Rattus norvegicus tümör hücrelerine doku kültüründe ^{3}H–timidin inkorporasyonuna otoradyografi yöntemi ile bakılmış morfolojik değişimler ise ışık mikroskobu ile takip edilmiştir.

Doku kültüründe % 5 KDS li mediumda inkube edilen hücrelerde 17 saatten sonra bazı morfolojik değişiklikler izlenmiştir. % 10 yoğunlukta KDS lı besi ortamında 39 saat inkube edilen hücrelerde DNA sentezi her iki hücre grubunda inhibe olmaktadır.

Doku kültüründe % 5 yoğunlukta ki KDS li besi ortamında 39 saat inkube olan RT hücrelerinde DNA sentezi % 9.02 iken kontrol tümor hücrelerinde DNA sentezi % 36.62, deney REF hücrelerinde DNA sentezi % 6.05, kontrol REF hücrelerinde % 40.10 oranında bulunmuştur.

Kurbağa deri sekresyonunun DNA sentezi inhibitor etkisi hücrelerin yeni besi ortamına konmasıyla kaybolmuştur. Preinkubasyon zamanına bağlı olarak hücrelerin DNA sentezi normale dönmüştür. Bu konunun kısa derlemeside de yapılmıştır.

INTRODUCTION

One of the most typical characterictics of animals is their capacity to adopt to physical, chemical and biological conditions in their environments. Although microorganisms are abundant throughout the nature, the interrelationships between animals and microorganisms under natural conditions are little investigated. Of course, the animals of economical and medical importances are excluded in this generalization. This subject–matter includes many interesting topics of physiological and evolutionary importance. Extracts or substances from animals (except mammals) with biological activities like antitumoral, antimicrobial, anticoagulant etc. are listed on Table I.

Frogs could survive in dirty places with microorganisms as well as in clean water or land. Since they have nude skin, their skin could be easily scratched when they are in motion. Their skin is covered with a thin mucous layer. Infection on their skin is almost never detected. This fact is probably do to the presence of antimicrobial substances on their skin or in their skin secretions. We have reported earlier the isolation of an extract with antibacterial activity from the frog Rana ridibunda (16). In our preliminary studies, fibrinolytic system inhibitor activity was also detected in this extract. In this study, this extract isolated from the skin secretion of the water frog Rana ridibunda is investigated for its antifungal activity and for its effects on Rattus norvegicus embryonic fibroblast cells (REFC) and chemically induced tumor cells (RTC) grown in tissue culture. Also purification of the active substances with the above listed biological activities which were found to be proteins is established.

Table - I

Animals Group, Class	Origin	Biological Activity	Active Substance	References
PROTOZOA **Flagellata** *Gongaulax catenella* (Dinoflagellata)	Crude extract	Neurotoxin	Saxitoxin I (STX)	12 33
Gongaulax tamarensis	Crude extract	Neurotoxin	Saxitoxin I (STX)	2 33
METAZOA **Coelenterata** *Physalia physalis* (Jelly fish)	nematocysts' venom	Proteolytic enzyme activitiy	Toxin	48
Stomolophus meleagris (Jelly fish)	nematocysts' venom	5' nucleotidase, hyaluronidase, phosphatase, phosphodiesterase, leucine aminopeptidase and protease activities	Toxin	88
Chironex fleckeri (Australian Jelly fish) *Chrysaora quinquecirrha* (Jelly fish) *Physalia physalis* (Jelly fish)	whole body extract	rises blood pressure, hemolytic, dermonecrotic and lethal effects		88
Chironex fleckeri (Jelly fish) *Chry guinquecirrha* (Jelly fish)	Tentacles, nematocysts suspensions	ionic calcium uptake inhibitor	Toxin	11
Actinia equina	Tentacles' extract	(lethal)	eguinatoxin (Highly basic thermolabile protein)	26
Actina equina	Tentacles' extract	lethal, causes bradycardia	eguinatoxin Protein	81
Sea anemones	whole body extract		Serotonin, homarine	66
Anemonia sulcate (Sea anemone)	whole body extract	Increases Na^+/K^+ ATP ase activity.	Polypeptide toxins ATXI (Mr:4702) ATXII (Mr:4935) ATXIII (Mr:2678)	3

Animals Group, Class	Origin	Biological Activity	Active Substance	References
Palythoa vestitus (Soft coral)	whole body extract	One of the most potent coronary vasoconstrictors known highly toxic	Polypeptide High Mr polypeptide eg. the deadly botulinus toxin	12 97
COLEMATA **I- Echinodermata** **Asteroidea :** *Asterias rubens* (Sea star)	whole body extract	Haemolytic activity	Saponin like substances	49
Asteroids (Sea stars)	whole body extract		Saponin and glycosides mostly linked to sterols	12
Asterias (Sea star)	whole body extract	Antimicrobial activity	Glycones	83
Asterias (Sea star)	whole body extract	Antimicrobial activity	Glycones, a glycones as cholesterol or pregnenolon type structure	37
Marthasterias glacialis	whole body extract		Saponin like structures Asterosaponins (glycosidic sulphates) Aglycones (Steroidal saponin)	49 93
Holothuridea : *Holothurians* (Sea cucumbers)	whole body extract		Saponin and glycosides mostly linked to sterols	12
Holothuria sp.	whole body extract	Antimicrobial and antitumoral activity	Glycones (glycosides)	83
II. MOLLUSCA **Gastropoda :** Non–marine mollusca	whole body extract	RBC agglutination (A, B, A + B, H erythrocyte haemaglutinin)	Aglutinin A, B, A+B, H blood group substances	65
Aplysia californica (Sea hares)	Hemolymph	Opsonin activity a mechanism of bacterial clearance by the sea hare involving aglutinin, an opsonin activity.	Aglutinin, lack bactericidine	43 90 60

Animals Group, Class	Origin	Biological Activity	Active Substance	References
Aplysia california (Sea hares)	Serum	Bacterial clearance, immune defense mechanism and agglutinating marine bacterial and vertebrate RBC	Bacterial aglutinin (Protein)	61
Aplysiidae (Sea hares)	Midgut gland	Water–soluble toxin : produces a transient hypotension bradycardia and apnoea, convulsion, respiratory distress. Ether–soluble toxin : hypertension in rats, vasoconinstrictor action, irritability vicious and severe flaccid paralysis.	Water–soluble toxin Ether soluble toxin	99
Helix pomatia	whole body extract	Inhibitor of aglutination of B erythrocytes	B substances	29
Helix hortansis (Garden snail)	whole body extract	RBC aglutination (B and H erythrocyte haemaglutinin).	Aglutinin (B, H substances)	64
Helix hortansis (Garden snail)	Egg white gland extract	RBC aglutination (B erythrocyte haema-glutinin)	Aglutinin (B blood group substances)	76 45
Patella vulgate	whole body extract	RBC Aglutination (A_1, A_2 and B erythrocyte haemaglutinin)	Aglutinin A_1, A_2, B blood group substances	64
Cassidaria echinophora	Buccal gland	Stimulates immune defense mechanism	Sulphur rich compounds (Low molecular weight)	25
Thais haemostoma (Clench) Sea snail	Hypobranchial gland extract	1st active component; produced a direct stimulatory effect on the blood pressure and heart actions. 2nd active component; as neuromuscular blocking agent depolarizing type.	2 active components	40

Animals Group, Class	Origin	Biological Activity	Active Substance	References
Cephalopoda : *Octopus bimaculates*	Serum	Aglutination	A blood group substance	70
Octopus vulgaris	Posterior salivary glands	Neurotransmitter	Octopamine, p–hydroxyphenyl, Ethanol amine	44
Cephalopoda sps.	whole body extract	Pain reducer effective on romatoid arthritis	Enteramine, Histamine Octopamine Tyramine	44
Lamellibranchiata : Oyster	whole body extract	RBC Aglutination (A and H erythrocyte haemaglutinin)	Aglutinin (A and H blood group substances)	15 89
Crassostrea virginica (oyster)	whole body extract	Aglutination	Aglutinin (Protein)	89 1 100
Pinctada martensi (pearl oyster)	whole body extract	Aglutination	Aglutinin A and H blood group substances	102
Velesunia ambiquas (mussel)	Hemolymph	Aglutination	H and P_1 blood group substances.	30, 65 41
Mytilus californionus (California sea mussel)	whole body extract	Neurotoxin	Saxitoxin I (STX)*	33
III.ARTHROPODA **Crustaceae :** *Panulirus argus* (sping lobster)	Hemolymph	Antibacterial activity	Bactericidin	23
Homarus americanus (Lobster)	Hemolymph	Antibacterial activity	precipitin	84
Homarus vulgaris (Lobster)	Digestive juice		Surface–active substances (Fatty acyl dipeptide)	39

* Saxitoxin I (STX) is also isolated from *g. catenella* (flagellate) **isolated** M. *californianus* and *Alaska* butter clam *saxidomus gigantus,* both feed on *G. catenella*

Animals Group, Class	Origin	Biological Activity	Active Substance	References
Crangon crangon *Pandalus jordani*	Hemolymph	Stimulated succinate oxidation in intact rat liver mitochondria in metabolic state 4	Eyes talk factor	82 84
Procamborus bicarinatus (crayfish)	Hemolymph	Antibacterial activity	opsonic factors	57
Procamborus clarkii (crayfish)	Serum	Aglutinates marines bacteria, chicken and rabbit RBC	Aglutinin (Protein)	56
Astacus leptodactylus (cray fish)	Digestive juices Hemolymph	Surface active substances RBC Aglutination	Fatty acyl–tauirine Aglutinin	39 17
Carsinus mediterraneus (crab)	Hemolymph	Fibrinolytic system activator-plasmin-like	Protein	95
L. polyphemis (Horses shoe crab)	Hemolymph	Aglutination	Aglutinin Protein Mr: 150,000, heat and pH sensitive.	56
Insecta : *Dytiscus*	pigidal gland	Antibacterial activity		50
Selenopis geminata	venom	Antibacterial activity		77
Periplanata	chitin	Antibacterial activity	Amino–phenol	9
Atta sexdens	venom	Antibacterial activity		51
Acheta domesticus	gut contents	a microsomal enzyme inhibitor. (Inhibition apparently results from solubilization of NADPH Cytochrom C reductase from microsomal membranes)	Proteolytic enzme	10
Coccionella	Hemolymph	Stain and indicator	Carmic acid	76
Vespa orientalis	Venom	Venom acts directly on mitochondrial function and ultra–structure of the mice kidney.		73

Animals Group, Class	Origin	Biological Activity	Active Substance	References
Bees	Venom	Antiarthritic action increases corticosteron secretion	Protein phospholipase A and hyaluronidase, mellitin and apamine (mast cell degranulating peptide) (MCD)	2 80
Carabidae	Venom	Defense mechanism	Formic acid, iso valeril aldehyde, asetic acid, tigric acid.	78
Notonecta	secretion	Antibacterial activity	p.hydroxibenzal-dehyde	76
Tenebrio molitor	whole body extract	Antibacterial activity		76
Forficula auricularia	whole body extract	Antibacterial activity		79
Julus terrestris	whole body extract	Antibacterial activity	Tolohydrochion, Athydrochion	
Plealeachi	Metatorax gland	Antibacterial activity	H_2O_2	
Araneidea : *Androcteurus* Australis hektor	venom	Neurotoxin paralyzes mammalia and insects		71
Latrodectus sps.	venom	Most potent biologic toxin. Venom act destabilization of cell membranes and degranulation of nerve terminals resulting in the release of neurotransmitters Hypertension, painful muscle spasm.		71
Latradectus mactans	venom	Neurologic symptom in muscle		71
Loxosceles reclus	venom	Coagulating mechanism activator		98
IV. VERMES **Polymera (analida) spinculoidea :** *Spunculid worms*	whole body extract	Antibacterial activity	powerful bactericidin factor	23, 24 42, 47

Animals Group, Class	Origin	Biological Activity	Active Substance	References
Dendrobaena venata	whole body extract	Antitumoral activity on the sarcoma tumor, in vivo		18
Ascaris lumbricoides	whole body extract	Antimicrobial and toxic activity		76
Amera (Scolecida): *Plathelminthes* *Turbellaria* *Trematodes* *Cestodes*	whole body extract	Antibacterial and cytotoxic activity	Acid mucopoly saccharid	76
Hirudo medicinalis	gland	Anticoagulant	Hirudin	76
FROGS AMPHIBIA	Skin secretion	Antimicrobial activity	Antimicrobial substances	62, 63 54
Bombina varigeata L.	Skin secretion	Antimicrobial activity Bufo toxins can be differentiated from other anura secretions by the content of cardio active steroids	Protein, basic polypeptides, aminoacid and 5-hydroxytriptamine	46
B. varigeata	Skin gland secretion	Bactericidal activity	Two nonpeptides, low Mr peptides	5 37
B. varigeata L.	Skin gland secretion	Bactericidal activity	Two oligopeptides	14
B. varigeata varigeata	Skin gland secretion	Bactericidal activity Bombesin causes contraction on the uterine and intestinal smooth muscles of several animals, increases arterial blood pressure and stimulates gastric secretion in dogs and chickens.	Bombesin	53
B. varigeata pachpus	Skin gland secretion	Bactericidal activity Bombesin causes contraction on the uterine and intestinal smooth muscles of several animals, incre ase, arterial blood pressure and stimulates gastric secretion in dogs and chickens.	Active fraction does not show any typical u.v. absorption band as does material containing aromatic groups and does not react with ninhydrine.	13

Animals Group, Class	Origin	Biological Activity	Active Substance	References
B. varigeata varigeata	Skin secretion	Antimicrobial and some biologic activity. It causes systemic hypertension, bradycardia, constriction of the renal mesenteric and cardiac activity.	Bombesin (Tetradecapeptide)	27
Bombina bombina	Skin secretion	Antimicrobial activity	Low Mr peptides	37
Salamandra sp	Skin secretion	Antimicrobial activity venoms are active to microorganisms and their use is to protect the skin of amphibia against infection.	Azosteroids	8 34 37
Salamandra maculasa	Skin gland secretions	Antimicrobial activity substances inhibits the growth of microorganism to various degrees. The toxins show their effects as they appear primarily in the cytoplasm and on the membrane in saccharomyces cerevisiae.	Alkaloids Samandarone Samandarine Samondaridine	35 36 68 37
Leptodactylus pentadactylus (South American frog)	Skin gland secretions	Antimicrobial activity	Alkaloids Spinaceamine 6–Methyl– Spinaceamine	19 20 68 36
Bufo spp.	Skin gland secretion	Pharmacological activity, Antimicrobial activity	(Cardioactive steroids.) Alkaloid: Bufotenine Alvarobufotoxin Cinobufotoxin Fowlerbufotoxin Gamabufotoxine Marinobufotoxine Regularo– bufotoxine Viridobufotoxine	46 34 35 36 13 54

Animals Group, Class	Origin	Biological Activity	Active Substance	References
Hyla arborea (European free frog)	Skin secretion	Pharmacological and antimicrobial activity	Proteins (pharmacologically active proteins)	46 12 68
Urodela spp. Triturus cristatus	Eggs extract	Aglutination	Aglutinin Various substances with biological activity	45
Phylobates aurotaenia	Skin secretion	Aglutination	Batrachatoxin	37
Columbian poison (Arrow frog)	Skin secretion	Antimicrobial activity and biological activity more toxic to mice	Batrachotoxin III	37
Tarieba torosa (California newt tetradon spp.)	Eggs extract	Antibacterial activity Tetratoxin protects the egg clusters against predators and microorganisms.	Toxins Tarichatoxin Tetratoxin	28 91 37
Dendrobates spp.	Skin secretion	Antimicrobial activitiy	Alkaloids Cis–perhydroquinoline	37
Dendrobates histriomcus	Skin secretion	Potentiates and blocks the indirectly elicited muscle twitch in a concentration dependent manner	Tricyclic alkaloid Gephyrotoxin (GyTx)	74
Dendrobatide (Neotropical poison)	Skin secretion	Antimicrobial activity	Alkaloids	21
Phyllomedusa sauvagei	Skin secretion	Two powerful opioid peptides	Dermorphin Dermenkephalin The peptides issued from a common biosynthetic precursor. Dermophin from the precursor involves several posttranslational steps.	56 75
Xenopis spp.	Skin secretion	Antimicrobial activitiy	Xenopsin (biological active octa peptide)	85

Animals Group, Class	Origin	Biological Activity	Active Substance	References
Xenopis leavis	Skin secretion	Antimicrobial activity	Xenopsin, Caerulein TRH, PGLA fractions	31
Xenopus laevis (South African frog)	Skin secretion	Antimicrobial activity	Xenopsin, Caerulein, TRH, PGLA a HPLC chromatograms showed the secretion to be a complex mixture with over 30 components at similar levels to the four peptides previously isolated from X. laevis.	31 32
Xenopus laevis	Skin secretion	Antimicrobial activity	Caerulein was isolated and sequenced using restriction endonuclease	72
Xenopus laevis	Skin secretion	Antimicrobial activity	Antimicrobial active peptides	103 101
Xenopus laevis	Skin secretion	Antimicrobial activity, antiprotozoal	Antimicrobial active peptides The sequence of a partial C.DNA of precursor reveals that both peptides derive from a larger protein. These peptides appear to represent a previously unrecognized class of vertebrate antimicrobial activities.	103 67
Xenopis laevis	Skin secretion	Antimicrobial activity, non hemolytic and antiprotozoal activitiy	Peptides Magainin 1 Magainin 2 Neurotensin (like octapeptide)	103

Animals Group, Class	Origin	Biological Activity	Active Substance	References
Xenopis laevis	Skin secretion	Antimicrobial activity	Antimicrobial peptides (Magainin)	58
Xenopis laevis	Skin secretion	Antimicrobial activity	Peptides Magaının 1 and 2, Levitidae, Xenopsin, Caerulin, Angiotensin, Bombesin, Bradykinin, Dermorphin, Sauvagıne, Spasmolysin, Tachykinins, Thyrotropin.	6
Rana spp.	Skin secretion	Antibacterial activity	Serotonin (as active protein) toxic substance (in some species)	54
Rana esculenta (water frog)	Skin secretion	Antimicrobial and Pharmacological activity	Pharmacological active peptides	46
Rana esculanta	Secretory gland neurointermediate pituitary	Antibacterial activity	Polypeptide	6
Rana ridibunda	Skin secretion	Antibacterial activity		16
Rana pipiens	Skin secretion	Antimicrobial activitiy	Neuropeptide The skin is a complex secretory tissue, but the presence of large quantities of TRH and other biologically active neuropeptides make this tissue an attractive model to study the regulation of neuropeptide secretion	7

Animals Group, Class	Origin	Biological Activity	Active Substance	References
SNAKE *Elapide*	Venom	Neurotoxin and cytotoxin	(Protein) Neurotoxin I Neurotoxin II Neurotoxin III Neurotoxin IV Toxin A Toxin α Toxin B Toxin Y Cobratoxin Toxicin F2 Toxicin F1 Toxicin F3 Toxicin E5 Toxicin E6 Toxicin E7 Principal neurotoxin Minor neurotoxin α Bungarotoxin Notexin	92 86 87 22
Hydrophiidae (Sea snakes)	Venom	Neurotoxin and cytotoxin	(Protein) Toxin a Toxin b Erabutoxin a Erabutoxin b	
Viperidae (viperids, vipers)	Venom	Neurotoxin hemolytic and Coagulant activities	Viperotoxin Toxin	83
Crotalidae (Crotalids, pit vipers)	Venom	Neurotoxin hemolytic and coagulant activities	Crototoxin Crotamin	
FISH *Anguilla*	Serum	Hemolytic and Ichthyotoxic activitiy	Ichthyotoxin, hemolysin cytotoxin	4
Rypticus saponoccus (Atlantic soapfish)	Cutaneous gland toxin Foamy secretion	Highly toxic to fish and mice	Protein, polypeptide	52
Pardachirus marmoratus (flat-fish)	Skin secretion	Hemolytic and Ichthyotoxic activitiy	Protein	69
Gobiodon spp.	Skin secretion	Hemolytic and Ichthyotoxic activitiy	Toxin	38

MATERIAL AND METHODS

Isolation of the crude skin secretion extract and purification of the biologically active proteins from the crude extract : The water frogs, *Rana ridibunda* were obtained from Alibeyköy and Çorlu in Istanbul. Under ether anestesia the dorsal skin of the frog was massaged with fingertips back and forth to situmulate the foamy skin secretion. The secretion was collected with a spatula into a test tube, left in water bath at 100°C for 30 min, centrifuged at 5500 rpm for 30 min (T30 Janetzled 30 type centrifuge). The supernatant was used as the crude extract (CE). CE was precipitated with 95% $(NH_4)_2SO_4$ cut. Centrifuged at 10,000 rpm for 10 min (Beckman 21 rotor). Precipitate was dissolved in and dialyzed against 0,9 % NaCl. The skin secretion was further purified by either sephadex G25 or Sephadex G75 column chromotography (column height : 25 cm, column with 10 cm). 1 mg/ml protein containing crude extracct was applied to the column. 1 ml fractions were collected. The elution was done with physiological serum. The absorption at 280 nm was recorded for each fraction. The protein fractions with the biological activities obtained from Sephadex G75 column chromatography was further purified by DEAE Sephadex A25 column chromatography using the same procedure as above except the elution was done with stepwise NaCl concentrations (0.02 M – 1.00 M). The purification procedure is summarized in Fig. 1.

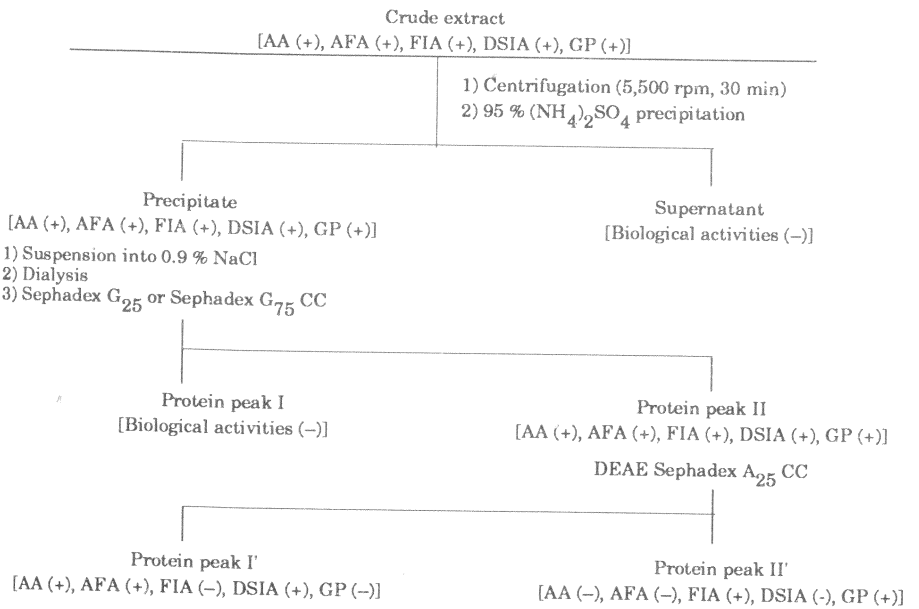


Fig. 1 : Purification steps of the skin secretion and biological activities present (AA : Antibacterial activity; AFA : Antifungal activity; FIA : Fibrinolytic system inhibitor activity; GP : Glycoprotein; DSIA : DNA synthesis inhibitor activity; CC : Column chromatography; (+) : Activity present, (–) ; activity absent).

Determination of antifungal effect : Fungi and mold were obtained from Istanbul University, Istanbul Medical School, Department of Microbiology. Sabouraud dextrose agar medium sterilized at 115°C in the autoclave for 15 min was used. After sterilization, 30 U/ml penicillin, 30 mg/ml streptomycine and 0.5 mg/ml actidion and 0.25 ml/ml skin secretion were added into the medium. Two ml aliquats were put into sterile tubes. The controls contained physiological serum instead of skin secretion. Fungi and dermatophyte strains were inoculated. The tubes inoculated with dermatophytes were incubated at 26°C for 10 days. The antifungal effect of the skin secretion was followed for each strain on the inoculated and incubated tubes by following the cell growth.

Isolation of *Rattus norvegicus* tumor cells (RTC) : Tumor was developed by chemical carcinogenesis. The neck skin was treated with either croton oil (H.J. Muller, Hamburg) or 7,12–dimethyl benzanthracene (Fluka AG. Buscks SG). 8 months later, the tumor developed was transferred into tissue culture medium under aseptic conditions. Passages were done at certain intervals into the medium containing 10% bovine serum where an antibiotic was also included. Tumor cells were kept after freezing in liquid nitrojen in deep freeze at –20°C until use.

Isolation of *Rattus norvegicus* embryonic fibroblast cells (REFC) : The embrionic cells were obtained from 10–15 days pregnant rats. The embrionic tissue was separated into its cells by trypsinization. Upon tissue culture procedure of the embriyonic cells, primary cell culture was obtained. The cultures obtained from the secondary passages were used in the experiments.

Investigation of the effect of frog skin secretion on morphology of *Rattus norvegicus* tumor (RTC) and the normal embryonic fibroblast cells (REFC): Four sterile cover–slip were put in each sterile petri dishes, 30 µl CE or protein fractions were put on the cover–slip and they were incubated at 37°C for 1 hr to establish the sticking of CE or proteins onto coverslips. Cell suspension (0.1 ml) (RTC or REFC) $5x10^5$ cells/ml was inoculated onto each coverslip, kept at 37°C for 30 min. Growth medium (10 ml) containing Eagle's minimal essential medium (MEM) with Earle's basic salt solution (EBSS), 2mM/ml glutamine, 100 units/ml penicillin, 35 units/ml neomycin, 50 units/ml mycostatin and with additional non essential amino–acids, 1.7 mg/ml

sodium bicarbonate and 20 % calf serum was added in petri dishes. Cells were incubated in petri dishes for 1 hr in an incubator containing 95% air and 5% CO_2 . Then the medium was withdrawn and 10 ml growth medium (GM) was added to each petri dish containing 5% or 10% CE or protein peak I' (8% , 4% , 2% , 1%) over cover slips including cells were incubated for various periodic times (1-72 hrs) at 37°C later the cover slips were removed from the medium having CE or protein peakI' medium. The cover slips were put on a self constructed special microscope slide chamber, having the cell growing surface inverted face downward on the holes (a special microscope slide chamber. 10 mm diameter x 1,5 mm depth) surface touching medium containing above mentioned percent ages of CE or protein peak I'. They were investigated under the phase–contrast microscope vitally and their microphotographs were taken at 40x10 enlargements.

Investigation of the effect of skin secretion on ^3H–thymidine incorporation into DNA of RTC or REFC (autoradiographic studies)

The cells were incubated in 5% or 10 % skin secretion containing GM in petri dishes at 37 °C for 39 hours. The cells were separated from the medium and washed with Basalt salt solution (BSS) and were transferred into 10 ml of culture medium containing 3 mCi/ml of ^3H–thymidine (Amersham Co. sp. act. 5000 mCi/mmole) incubated at 37° C for 30 min. The coverslips were fixed with acetic acid : ethanol (1:3) for 15 min, washed for 5 min with 70 % ethanol, dried with air and the coverslip containing these cells were mounted on gelatinized microscope slides. Then covered with Kodak AR–10 stripping film in a dark room. They were placed in black light–tight boxes together with some silicagel and a small amount of solid CO_2 to remove O_2 . The boxes sealed with black tape and stored at +4 °C for one week for exposure. All procedures were carried out in safe light illumination. Also all the developing procedures were carried out in safe light illumination. The slides were allowed to reach room temperature then developed at 18–19 °C by placing in D_{19} for 2 1/2 minutes and washed in 5% acetic acid for 1/2 min. The slides were then transfered to 1/4 fixol for 10 minutes. Following washing by standing in water for 10 minutes, they were drained and dried. The slides were stained with a diluted solution of Giemsa blood stain containing citric acid, $NaHPO_4$, methyl alcohol and Giemsa stock. The slides were stained for 7 minutes at room temperature. Then dipped

twice in distilled water and dried at room temperature. The slides were then examined by oil immersion for the presence of silver black grain. According to the intensity of the incorporation of radioactivity, the labelling of the cells were grouped as very little labelled, little labelled or highly labelled and embrionic fibroblast cells and tumor cells were counted according to the intensity of ^3H–thymidine labelling.

Recovery studies : The cells were incubated in a medium containing CE (5% or 10%) at 37 °C for 17 hrs. Then washed 3 times with BSS and transferred into a growth medium without skin secretion (CE) and incubated at 37 °C for 22 hrs. As the positive control, cells were incubated in a medium with 5% CE for 39 hrs. As the negative control, cells were incubated in growth medium without skin secretion (CE) for 39 hrs. The cells used were either RTC or REFC mentioned above.

The same procedure was repeated to show the effect different incubation times on recovery where 17, 39, 48 and 72 hours of incubation times were chosen. The negative and positive controls with respective incubation times were also included and followed until the cells showed monolayer growth.

RESULTS

Purification of skin secretion : In Fig. 1 together with purification steps the biological activities and presence of glycoprotein are sumarized. $(NH_4)_2SO_4$ precipitated (95 % cut) protein fraction of the crude extract (CE), isolated by centrifugation of the boiled skin secretion supernatant, was applied either to Sephadex A25 or Sephadex G75 and to DEAE Sephadex A25 column chromatographies. The biological activities namely antibacterial (AA) to Gram negative and Gram positive bacteria, antifungal (AFA), DNA synthesis inhibitor (DSIA) and fibrinolytic system inhibitor activities (FIA) determined with euglobulin lysis time and fibrin plate methods were all detected in the protein peak II which comes out after the void volume which implies molecular weights under 25,000 Da's. In protein peak I' all of the above activities except FIA were detected. FIA was detected in protein peak II'. Glycoprotein structure was found only with FIA.

The elution profile of Sephadex G75 column chromatography is seen at Fig 2 . The biological activities were all positive in fractions 21, 22 and

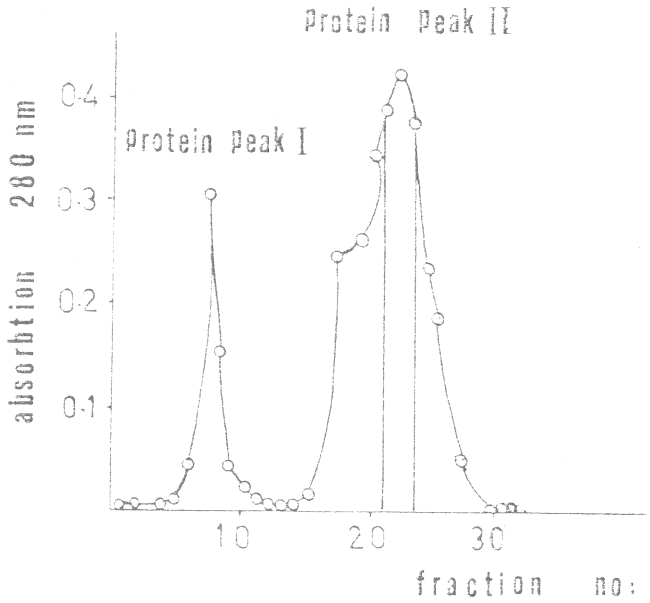


Fig. 2 : Sephadex G_{75} column chromatography of $(NH_4)_2SO_4$ precipitated frog skin secretion (FSS).

23. These fractions were poled together, concentrated with Amicon ultrafiltration using PM 10 membranes and passed through DEAE Sephadex A25 column chromatography, (Fig. 3) The protein peak I

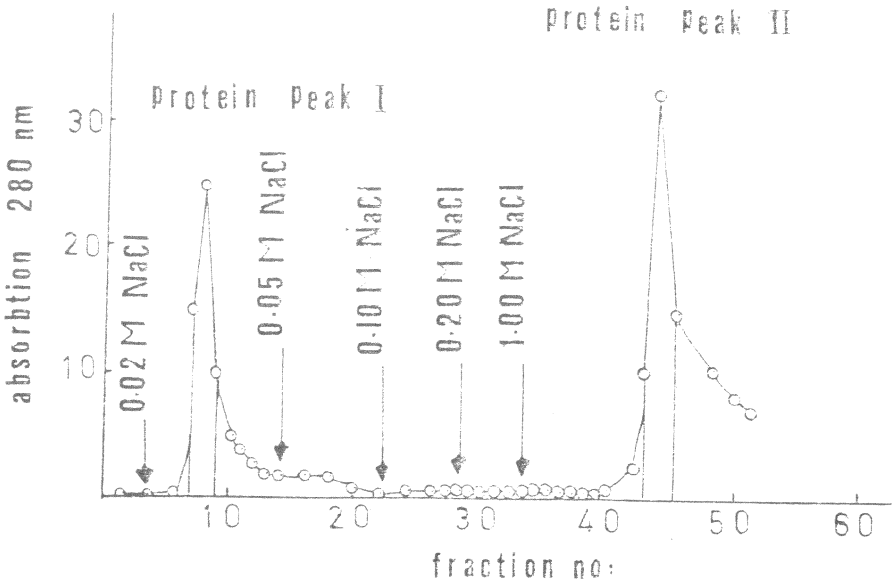


Fig. 3 : DEAE Sephadex A25 column chromatography of pooled protein peak I

appearing with 0.02 M NaCl elution had both the AA and AFA together with DSIA. The protein peak II' appearing upon 1 M NaCl elution had the FIA. Throughout the purification steps the GP presence was also checked with periodic acid shiff (PAS) reagent and the glycoprotein structure was found to be related with fibrinolytic system inhibitor activity. The summary of the purification steps and spesific antibacterial activities at each step are summarized at Table II to show the efficiency of purification at each step. After DEAE Sephadex A25 column chromatography step, both proteins I' and I" showed a single protein band upon cellulose acetate electrophoresis at pH 8.6. It looks as if all three biological activities namely antibacterial, antifungal, antitumoral activities are due to a protein with a molecular weight between 10,000 and 25,000 Da.

Table – II : The efficiency of purification followed with antibacterial activities upon Staphyloccus epidermidis 69 micrococcus using hole method (16).

Method	Spesific activity inhibition zone (cm/mg)	Purification fold	Total before			Total after		
			Volume (ml)	Protein (mg)	Activity unit (inhibition zone cm)	Volume (ml)	Protein (mg)	Activity unit (inhibition zone cm)
Crude extract	1.9	–	29	1177	2236	–	–	–
95 % $(NH_4)_2$ SO_4 ppt.	4.7	2.5	20	1030	2000	22	141	661
Sephadex G75 CC	8.4	4.6	20	126	600	48	58	484
DEAE Sephadex A25 CC	12.2	6.4	45	54	453	40	37	451

The effect of skin secretion on fungi : The antifungal effect is seen on Table III. The skin secretion was effective on all of the fungi tested except to Torulopsis glabrata. All of the strains grew on control Sabouraud medium.

Table - III : The effect of frog skin secretion on the fungi strains obtained from İstanbul University, Istanbul Medical Faculty, department of Microbiology (AFA).

Fungus	Code no.	Frog skin secretion.	Control
Yeasts			
Candida albicans	7650	–	+
C. parapsilosis KUEN	1010 (Y) C1–12–1	–	+
C. krusei	KUEN1001 (Y) C1–6–3	–	+
C. albicans klaur	A–2	–	+
C. albicans		–	+
C. tropicalis	KUEN1022 (Y) C1–19–3	–	+
C. stellatoidea	KUEN1018 (Y) C1–18–1	–	+
C. albicans	MIV–211	–	+
Cryptococcus neoformans	KUEN1047 (Y) C2–1–9	–	+
Torulopsis glabrata		+	+
Rhodotorula glutinis	KUEN1064 (Y) R1–1–1	–	+
Saccharomyces cereviseae		–	+
Mould			
Microsporum nanum	KUEN1089 (F) M5–6–1	–	+
Trichopyton mentagrophytes	703	–	+

+ : growth was seen

– : no growth

The effect of frog skin secretion on the morphology of Rattus norvegicus tumor (RTC) and normal embryonic fibroblast cells (REFC) :

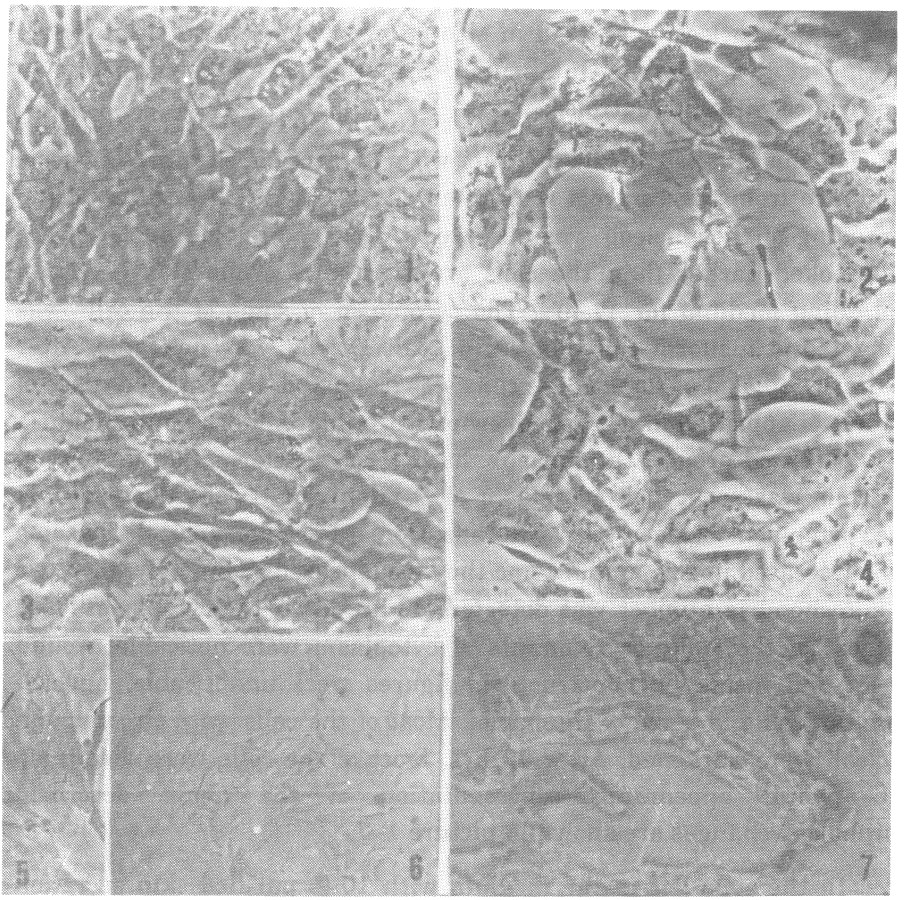
In the cells left in the medium with 5 % crude skin secretion, no differences from controls were observed in 1 and 3 hrs of incubation. After 17 hrs of incubation, some differences like darkining of the nucleolus were observed. After 26 hrs, further darkining of the nucleolus and decrease in cytoplasm volume of some of the cells were observed but still the cells were alive. After 39 hrs 1 decrease in the cell number was seen. The individual cells became more circular and sharper, appearence of nuclei were noticible. 48 hrs later most of the cells were circular and their nuclei appeared darker. 72 hrs later most of the cells were circular,

some of the nucleoli decreased in size and they were disintegrated, the darking of the cells was intensified. Still there were cells similar in appearance with the controls.

In the cells left in the medium with 10 % crude skin secretion, no difference from the controls was observed in 1 and 3 hrs. After 6 hrs, darkening of the nucleoli was observed. After 17 hrs, the nuclei of the cells looked darker than the cells grown in 5 % crude skin secretion containing medium for 17 hrs. The cytoplasm of some of the cells was alongated in fusiform shape but still healthy cells were noticable. After 26 hrs, cytoplasm of some of the cells was elongated, the circumference of the nuclei appeared like it was drawn with Indian ink. The mitochondria were gathered around the nucleus. 39 hrs later, deformation in most of the cells was seen. The cytoplasm of the individual cells decreased in size, cytoplasm bridges between cells were noticable, nucleoli in some cells decreased in size and in some they disappeared completely. Increase in mitochondria number was detected. 48 hrs later, the cytoplasm lysed in most of the cells, or remnants of cytoplasm were noticable around nuclei and inside nuclei structural figures were unnoticable, nucleoli were noticable in some of the nuclei. Most of the cells were circular and nucleoli were darkened. After 72 hrs, most of the cells were deformed. The typical appearance representation of the above described morphology changes are shown in picture 1–7.

In the cells left in the medium with 10 % purified skin secretion (protein peak II of Sephadex DEAE–A25 column chromatograhy) no difference from the controls was observed in 1 and 3 hrs. After 6 hrs, the nuclei of the cells looked darker. After 17 hrs, decrease in cell number was observed. Some of the cells stood up on the coverslip and decrease in the cytoplasm and vacuolization was observed. 24 hrs later most of the cells died, the little number of cells sticking onto the surface with fusiform structure were noticable.

In the cells left in the medium with 8 % purified skin secretion, no important differences from the controls were noticable in 1,3 and 6 hrs. After 17 hrs, some of the cells had decreased cytoplasm 24 hrs later the nucleoli in some of the cells were darkened, decrease in cytoplasm in some of the cells was noticable.



Picture 1 : Tumor cells kept for 17 hrs in CE containing medium

Picture 2 : Tumor cells kept for 39 hrs in CE containing medium

Picture 3 : The control cells (non treated tumor cell with CE)

Picture 4 : Tumor cells kept for 17 hrs in CE containing medium and transferred to new growth medium (after recovery)

Picture 5 : The control cells (non treated embrionic cells with CE.

Picture 6, 7 : The normal embryonic cells kept for 39 hrs in CE containing medium.

Most of the cells left in the medium with either of 4 %, 2 % or 1% CE no differences from the normal controls were noticable upto 24 hrs.

When the cells were incubated for 24 hrs in 10 % skin secretion, they recovered after 4 days, when incubated for 39 hrs, they recovered

after 6 days, when incubated for 48 hrs, they recovered after 9 days, when incubated for 72 hrs, they recovered after 12 days. The recovery time was elongated proportionally to the incubation time in a medium with skin secretion. (Fig 4)

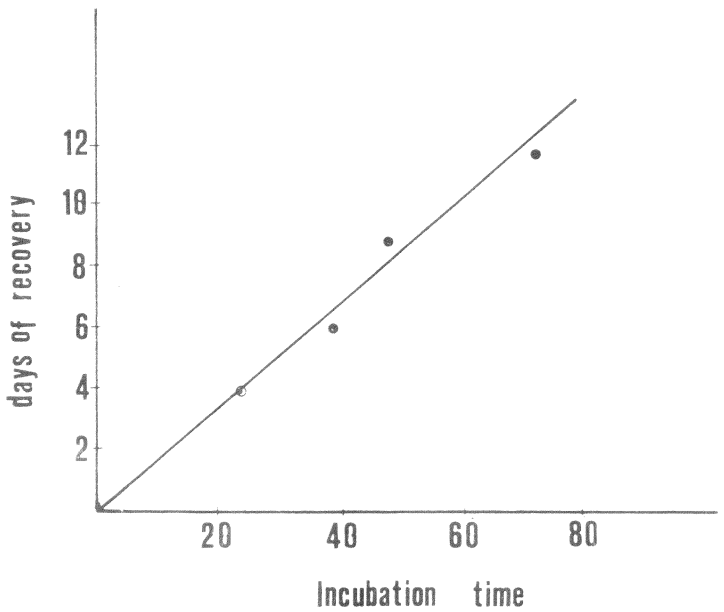


Fig. 4 : The effect of incubation time in a medium with 10 % skin secretion on recovery days after transfer into new growth medium without skin secretion.

The effect of skin secretion on ^{3}H-thymidine incorporation in DNA of the RTC and of REFC : Thymidine incorporation was detected in 2.75 % of the RTC grown in 5 % skin secretion containing medium. This percentage was 1.35 % for embryonic fibroblasts. In the tumor cells at 39 hrs of incubation in the medium without skin secretion this percentage was 27.95 %. It was 34.15 % for the embryonic fibroblast cells.

The cells grown in the medium with 10 % skin secretion, the DNA synthesis was 0 % for both kinds of cells at 39 hrs. After incubation of cells for 17 hrs with 5 % skin secretion they were transfered into normal medium ^{3}H-thymidine incorporation in tumor cells after 22 hrs of

recovery the percentage was 13.55 %. This ratio was 17.75 % for embryonic fibroblast cells, the cells kept in the medium having 5 % skin secretion for 39 hrs (as control) ^{3}H–thymidine incorporation for RTC were 2.75 % and it was 1.35 % for embryonic fibroblast cells (Fig 5, Fig 6)

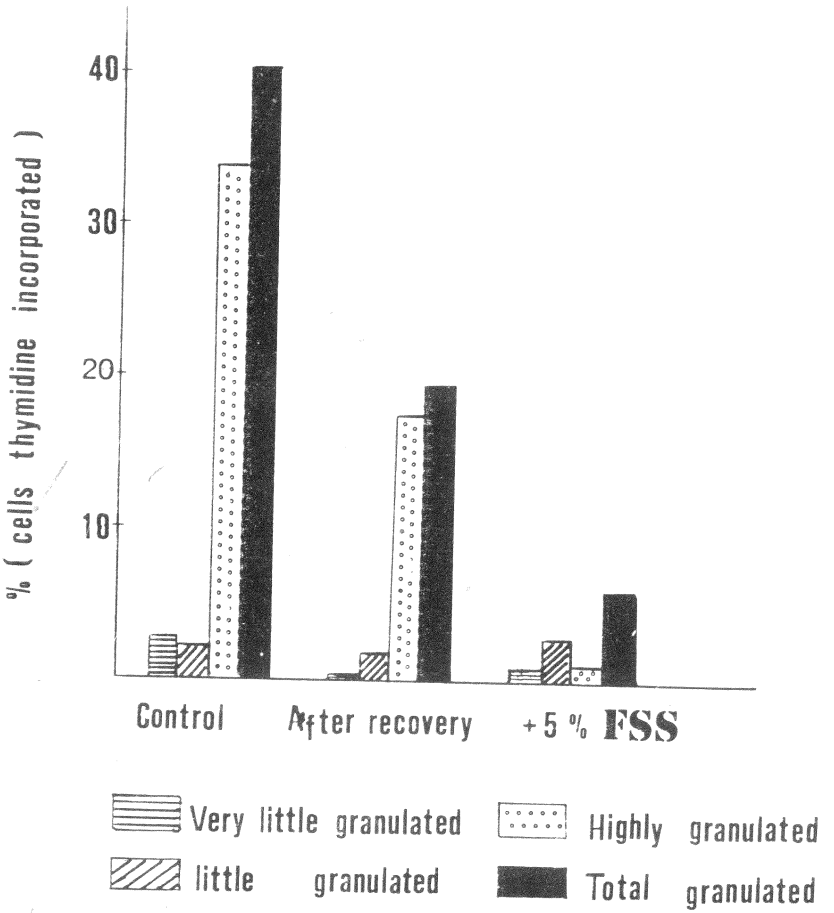


Fig 5 : The effect of skin secretion on DNA synthesis of Rattus norvegicus embryonic fibroblast cells. Thymidine incorporation is represented by the intensity of granulation.

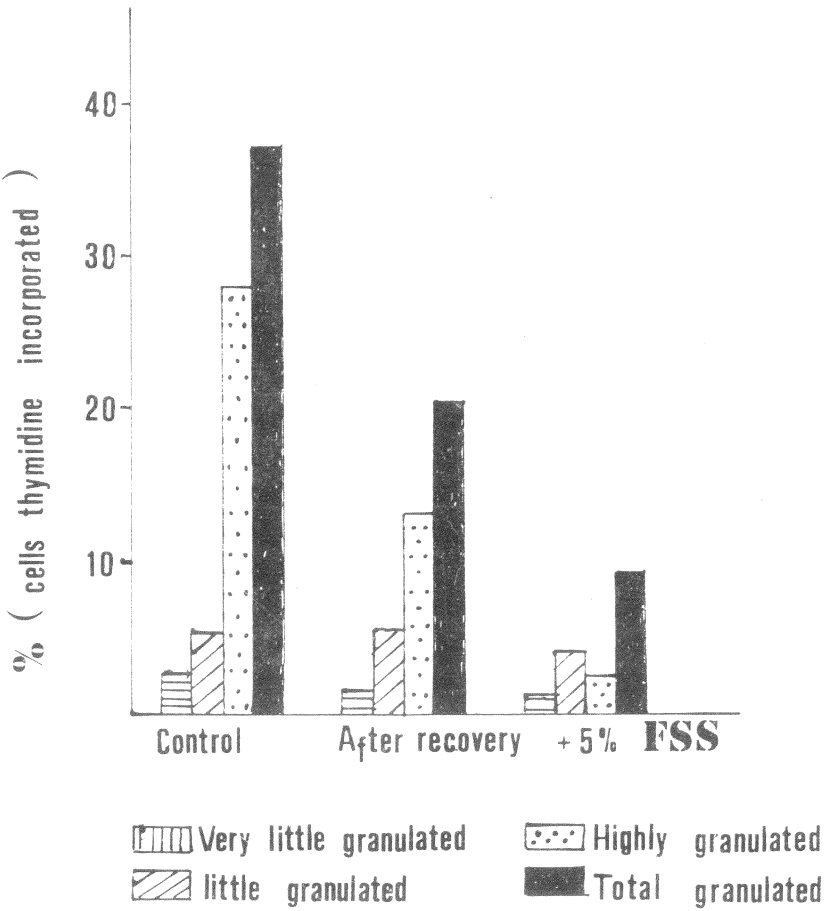


Fig 6 : The effect of skin secretion on DNA synthesis of *Rattus norvegicus* of tumor cells. Thymidine incorporation is represented by the intensity of granulation.

DISCUSSION

Various substances from different animal species with biological activities are reported in the literature. It is possible to detect these substances in protozoa coelenterata, coelamata and in the lower vertebrata like fish snake and amphibia (Table I). As seen at this table, most of the subtances with biological activities are of protein origin. Although there are quite a number of articles reported in the literature besides our work on amphibia, we believed that it would be very interesting to isolate substances with biological activities from the

extracts isolated from frogs of Turkey. With this intention, we perfomed this study.

Amphibian skin and skin secretions are well known as a rich source of biologically active substances. Mostly steroids and alkaloids with antimicrobial activities are reported (Table I). Peptides and proteins of amphibia are also reported to be a group of promising bioactive substances (6). A novel peptide Levitidine with a neurohormon–like activity was isolated from the South African frog Xenopus laevis by noradrenalin stimulation (67). Its amino acid sequence was determined and the complete c–DNA sequence of its precursor was accomplished. The other bioactive peptides isolated from the same source were cerulein, xenopsin and thyrotropin–releasing hormone. Levtide was found to be similar to alytesin, an amphibian skin peptide belonging to the bombesin family. Other peptides known as PGS peptides in Xenopus were reported to have potent antimicrobial activities. The amphibian sources and mammalian analogues of the peptides like angiotensin, bombesins, bradykinin, caerulein, dermorphen, sauvagine, spasmolysin, tachykinins, thyrotropin releasing peptide, trytophilin, and xenopsin are described in the review article 103 (103).

In this study, Rana ridibunda skin secretion was investigated with respect to biological activities. A protein with antimicrobial, antifungal activities and glycoprotein with fibrinolytic system inhibitor activity were isolated. These proteins were found to be heat stable.

The antimicrobial activity detected in Rana ridibunda skin secretion belonging to a protein is inaccordance with the literature findings of other amphibia species (16).

This protein with M of 10,000 – 25,000 and with antibacterial and antifungal activities was found to be nontoxic to normal embryonic fibroblast cells and to tumor cells in vitro at the doses of antimicrobial effects. At very high doses, the thymidine incorporation into tumor and normal fibroblast cells of Rattus norvegicus were inhibited but when transferred to a medium without the Rana ridibunda skin secretion protein, the recovery of the DNA synthesis was established and they had a strong potency for becoming medically important substances. The most important advantages of these proteins are the antibacterial activity to a large spectrum of microorganisms and the fibrinolytic system inhibitor activity appearing in another molecule.

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