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FLOWER POLLEN EXTRACT

STEM CELL SUPPLEMENTS

Pollitin is a high quality natural extract. extracted from rye pollen under the production and research with technology The same standard as the production of drugs according to the requirements of the World Health Organization. therefore has been registered as “NUTRACEUTICAL” or “nutritional therapeutic nutrition” receiving the ORAC standard or the antioxidant concentration and the CAP-e Test or the ability to be absorbed into red blood cells at a very high level

The body receives almost 100% of the nutrients that are extracted from rye grass pollen. Sold to more than 50 countries on 6 continents around the world for more than 50 years, Swedish researchers have found that research studies. extracted from rye pollen contains Substances that are essential for the creation of new life in the plant family and are fundamental in the food chain. It is a natural anabolic steroid.

It has been proven by scientific laboratories that Contains a variety of nutrients including vitamins, minerals, phytosterols, carotenoids, flavonoids, nucleic acids, amino acids, substances necessary for the synthesis of RNA and DNA, antioxidant activity, enzymes, saturated fatty acids, precursors in the synthesis of prostaglandins.

So extracted from rye pollen Therefore, it is the ideal food for use in helping to make the body healthy and perfect holistic. Because there are nutrients that help to relieve fatigue, have antioxidants. The main culprit that causes many serious diseases to humans, contains important substances such as phytosterols that help boost immunity. keep the body healthy until able to cope with various illnesses caused by facing pollution and germs on a daily basis more effectively

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Research has confirmed that there are more than 300 types of nutrients, vitamins, minerals that are essential for the care of the body and cells.

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Contains important substances that have antioxidant properties. Thus helping to slow down aging and help your skin look better.

BODY IMMUNE DEFENCE

Research reports on efficacy that helps to inhibit prostatitis caused by hormones

PHARMACEUTICAL FOOD

Contains nucleic acids and other important substances that stimulates the body to create interferon to stimulate white blood cells to work more efficiently better deal with germs

GUARANTEED WORLD-CLASS PRODUCTION STANDARDS

Natural Choice Nutraceuticals

POLLITIN - EXCLUSIVE STEM CELL SUPPLEMENTS

Our premium natural extracts originate from meticulously selected flower pollen found in "Rye." These extracts undergo a unique proprietary production process crafted by Graminex L.L.C. in Ohio, United States. This exclusive process encompasses every stage, from cultivation and harvesting to the creation of high-quality natural extracts, specifically G60 and G63, derived from GBX flower pollen particles. Graminex holds the sole rights to this process and maintains adherence to strict pharmaceutical production standards in alignment with the World Health Organization's requirements.

Our extracts are renowned for their world-class production standards, boasting ORAC certification for exceptionally high antioxidant concentration and CAP-e Test accreditation, which signifies outstanding absorption into red blood cells. Over more than five decades, we have consistently refined and improved our product's efficacy.

Registered as a "NUTRACEUTICAL" or "nutritional therapy," Pollitin addresses issues at the cellular level, offering antibacterial properties and reinforcing immunity. By delivering essential nutrients tailored to various bodily systems, it equips the body to effectively combat abnormal cells. Our dedication to research is exemplified by over 150 certifications from medical and pharmaceutical institutions.

Moreover, Pollitin is not only a national achievement but a global triumph, available in over 50 countries. Our exclusive patented production process sets us apart as the sole producer of this unique formulation globally, rendering it impossible for anyone else to replicate our success in extracting and utilizing these flower pollen particles.

Pollitin - สารอาหารบำบัดเซลล์อ

สารสกัดธรรมชาติคุณภาพสูง สกัดจากเกสรดอกไม้จาก “ข้าวไรย์” ที่มีสูตรลับเฉพาะของ บริษัท (Graminex L.L.C.) ที่รัฐโอไฮโอ้ ประเทศสหรัฐอเมริกา ในการปลูก เก็บ และผลิตสกัดธรรมชาติคุณภาพสูง G60, G63 จากอณูละอองเกสรดอกไม้ GBX, Graminex® เอกสิทธิ์เฉพาะของบริษัท Graminex เท่านั่นที่ผลิตได้เพียงเจ้าเดียวในโลก อยู่ภายใต้การควบคุมมาตรฐานการผลิตยา ตามข้อกำหนดขององค์การอนามัยโลก

จนเราได้รับการรับรองมาตรฐานการผลิตระดับโลกระดับเดียวกับการผลิตยาเพราะ Pollitin ได้รับรองการทดสอบค่า ORAC หรือ ค่าระดับความเข้มข้นของสารต้านอนุมูลอิสระที่สูงมาก และ CAP-e Test หรือค่าความสามารถในการดูดซึมเข้าสู่เม็ดเลือดแแดงในระดับที่สูงจนได้รับ

การขึ้นทะเบียนเป็น “NUTRACEUTICAL” หรือ “โภชนเภสัช สารอาหารบำบัดระดับเซลล์” ที่สามารถแก้ไขปัญหาฟื้นฟูได้ลึกถึงระดับเซลล์ มีฤทธิ์ฆ่าเชื้อแบคทีเรีย และมีผลเสริมสร้างภูมิต้านทานเมื่อเซลล์ต่างๆ ได้รับสารอาหารที่เหมาะสมตามระบบต่างๆ ในร่างกาย ส่งผลให้ร่างกายสามารถต่อสู้กับ เซลล์ที่ผิดปกติภายในร่างกายได้ถึง 95% และยังได้รับรองมาตรฐานการผลิตและประสิทธิภาพจากองค์กรต่างๆ มากมายระดับโลก รวมไปถึงยังได้รับรางวัลการันตีอีกมากมายจาก เอกสิทธิ์สูตรลับพิเศษเฉพาะของ Graminex ทำให้สินค้ามีคุณภาพและเกิดผลลัพธ์ที่ดีและน่าเชื่อถือ จนได้รับการยอมรับระดับสากลอีกด้วย

ตลอดระยะเวลากว่า 50 ปี เราได้มีการวิจัยพัฒนาประสิทธิภาพอย่างต่อเนื่อง มีการวิจัยจากสถาบันทางการแพทย์และเภสัชกรรมรับรองมากกว่า 150 การวิจัย เรามีความภูมิใจอย่างมากในการเป็นผู้ผลิตหนึ่งเดียวของโลกที่ได้ครอบครอง ถือลิขสิทธิ์เอกสิทธิ์กระบวนการผลิตและสูตรเฉพาะ G60 และ G63 จากละอองเกสรดอกไม้ชนิด GBX ที่ไม่มีใครสามารถทำได้ ส่งผลให้ Pollitin เป็นที่ยอมรับจากคนจำนวนมากใน 6 ทวีป 50 ประเทศทั่วโลก และได้รับผลตอบรับที่ดีจากผู้บริโภคในการซื้อซ้ำสินค้าอย่างต่อเนื่องมากกว่า 50 ปี

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FERTILITY SUPPORT

GRAMINEX Flower Pollen Extract

Efficacy of Cernilton administration for infertile males associated with asymptomatic pyospermia

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Introduction

The cases, that white blood cell is significantly higher in semen, accounts for 16 ~ 17% of male infertility patients. Interestingly, it was common that no bacterial finding is presented in these cases, using standardized bacterial test, PCR methods for Chlamydia trachomatis (C. trachomatis), and semi-quantitative analysis for Ureaplasma urealyticum (U. urealyticum). Although these cases are classified in nonbacterial chronic prostatitis, it has been generally recognized to be associated with male infertility.

In present study, we reported that administration of Cernilton reduce PMN-elastase activity and to improve seminal findings in semen for 17 male infertility patients with no bacterial finding in semen.

Materials and Methods

17 male infertility patients associated with nonbacterial asymptomatic pyospermia were treated with Cernilton 6 tablets daily over 12 weeks, and then sperm density, progressively motile sperm ratio, sperm motility and PMN-elastase activity in semen were measured.

Results

In all patients, progressively motile sperm ratio, sperm motility and PMN-elastase activity in seminal fluid were improved.

Conclusion

Administration of Cernilton is seemed to be effective in the treatment of infertile males associated with nonbacterial asymptomatic pyospermia

List of Patients

Number	Age	Sperm Density ($\times 10^6$/ml)			% progressively motile sperm			Sperm motility			PMN—elastase activity (µg/ml)		
1	35	31	→	59	34	→	42	±	→	+	760	→	529
2	33	44	→	40	31	→	52	±	→	++	1770	→	640
3	40	22	→	24	40	→	48	±	→	+	2080	→	610
4	32	10	→	10	30	→	54	±	→	+	7130	→	1860
5	36	38	→	27	42	→	55	±	→	+	610	→	240
6	34	32	→	35	40	→	52	±	→	+	890	→	290
7	35	28	→	31	44	→	67	±	→	++	2270	→	114
8	36	67	→	49	41	→	62	±	→	++	1710	→	380
9	41	100	→	64	15	→	55	±	→	+	1910	→	780
10	32	32	→	30	32	→	36	±	→	+	1510	→	1090
11	36	57	→	60	20	→	48	±	→	+	1800	→	1020
12	28	38	→	32	24	→	30	±	→	++	2820	→	980
13	30	1.2	→	1.6	0	→	20		→	+	3970	→	1120
14	36	0.3	→	0.3	40	→	50	±	→	+	1710	→	940
15	32	0.6	→	0.5	21	→	34	±	→	+	5140	→	1200
16	34	38	→	30	0	→	23		→	+	4520	→	1070
17	41	0.7	→	0.5	34	→	38	±	→	++	1060	→	720

MENOPAUSE SUPPORT:

GRAMINEX Flower Pollen Extract

Findings on Female Menopausal Disorders through the "Pollen Extract G63" of Graminex Company

Hiromi Yokoyama Naofumi Suzuki Yoshimi Nishimura

(Kanda New Medical Clinic)

Female Menopausal Disorders occur at the onset of menopause, have as a characteristic of indeterminate complaints, interference even occurs with intercourse, and becomes a source of discord in partner relationships. A reduction of female hormones has been talked about as the cause. Here, we have examined the influence of pollen extract G63 on hormones and improvement of the associated indeterminate complaints.

Objective and Methods

Six females, four in menopause and two evidencing menopausal symptoms having menstrual period every 4~5 months, were studied for degree of improvement according to two hormones Estradiol and DHEAS and the consultation questionnaire. The period of the trial was from 1 to 3 months. The pollen extract used in the trial was produced by Graminex Company in Ohio, USA from the pollen of raw materials such as rye, corn, and timothy hay (referred to as Phleum pratense in Japan) which were cultivated without using agrochemicals or genetically modified varieties. The pollen which has a double hull is not digested or absorbed even when ingested since it has strong resistance to acid and heat (cannot be destroyed even at 300°C). Graminex Company using a special technology is able to separately extract G60 (water soluble nutrition components) and GFX (lipid soluble components) and we received the product G63 which is a 20:1 combination G60 and GFX.

The dosage was 6 tablets per day; three tablets each taken after breakfast and dinner. One 250 mg tablet contains 62.5 mg of pollen extract.

(The daily quantity .... 375mg as pollen extract)

Our own medical questionnaire was prepared and the number of points evaluated. (Lower points indicate milder symptoms)

No.	Symptoms	None	Slightly Present	Medium Intensity	High Intensity
1	Heat sensitivity (burning sensation, hot flashes)	0	1	2	3
2	Chilling, numbness, edema of hands or feet	0	1	2	3
3	Perspiration	0	1	2	3
4	Tachycardia (rapid heartbeat)	0	1	2	3
5	Palpitation	0	1	2	3
6	Chest pains and breathlessness	0	1	2	3
7	Headaches	0	1	2	3

8	Feel heavy-headed	0	1	2	3
9	Insomnia	0	1	2	3
10	Depression	0	1	2	3
11	Irritability	0	1	2	3
12	Feeling of anxiety	0	1	2	3
13	Dizziness	0	1	2	3
14	Feel dizzy upon standing	0	1	2	3
15	Tinnitus (ringing in ears)	0	1	2	3
16	Stiff shoulders	0	1	2	3
17	Arthralgia in hands and feet	0	1	2	3
18	Lumbago	0	1	2	3
19	Numbness	0	1	2	3
20	Sensation like ants crawling on the skin	0	1	2	3

Results
Graminex Pollen Therapy Trials ... Female Menopausal Disorder

Name	Age	Examination day	Estradiol	DHEAS	Consultation questionnaire
O_T	48	-Before administration -After 2 months	Less than 10 Less than 10	65 83	17 21
Y_T	53	-Before administration -After 1 month	Less than 10 Less than 10	72 89	13 6
S_M	54	-Before administration -After 1 month	14 Less than 10	142 112	3 4
F_N	50	June 21, 2005	27 20	106 79	4 2
N_A	63	June 21, 2005	Less than 10 Less than 10	121 123	5 2
K_H	48	June 22, 2005	24 Less than 10	65 74	15 10

Conclusion

The increase in Estradiol was 0 for all subjects. DHEAS increased in 4 out of the 6 subjects and the average was 14.2%. Indeterminate complaints improved in 4 of the 6 subjects for a 54.1% degree of improvement.

Discussion

It can be considered that the improvement observed in indeterminate complaints was due to the amino acids, vitamins, and mineral components of the pollen extract which in the body assisted the promotion of metabolism. Additionally, there is evidence of rejuvenation with secretion of DHEAS which normally peaks for persons in their twenties. Moreover, the DHEAS value is also used as an indicator of female sexual desire and it can be considered that sexual appetite was also increased and it can be assumed that increased DHEAS helps to remove interference to intercourse for menopausal females.

Safety

Among the findings, in particular there were no side-effects and the supplement can be administered with peace of mind.

8/23/2005

OTHER SUPPORT:

GRAMINEX Flower Pollen Extract

A new herbal combination, Etana, for enhancing erectile function: an efficacy and safety study in animals

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We present herein a new herbal combination called Etana that is composed of five herbal extracts including *Panax quinquelotius* (Ginseng), *Eurycoma longifolia* (Tongkat Ali), *Epimedium grandiflorum* (Horny goat weed), *Centella asiatica* (Gotu Kola) and flower pollen extracts. Most of the above-mentioned extracts have been a long historical and traditional use for erectile dysfunction (ED). On the basis of the mechanism of action of each of the above, a combination is introduced to overcome several physiological or induced factors of ED. This study was conducted to show an enhancement of erectile function in male rats. The animals were observed for 3 h after each administration for penile erection, genital grooming and copulation mounting, and the penile erection index (PEI) was calculated. The maximum response was observed at the concentration of 7.5 mg kg^{-1} of Etana. At a 7.5 mg kg^{-1} single dose, the percentage of responding rats was 53 ± 7 with a PEI of 337 ± 72 compared with 17 ± 6 with a PEI of 30 ± 10 for control animals. This PEI was significantly ($P<0.001$) higher than each single component and than the sum of any two herbal components of Etana. When compared with sildenafil citrate, Etana induced more pronounced PEI than 0.36 mg kg^{-1}, but similar to 0.71 mg kg^{-1} of sildenafil. Furthermore, full acute and sub-acute toxicity studies showed no toxic effects of Etana. In conclusion, this study describes a new and safe combination of herbal components that enhance erectile function in male rats. Clinical studies are warranted for evaluating Etana's significance in ED.

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Keywords: Ginseng; Centella; Epimedium; Eurycoma; pollen; erectile dysfunction; Etana; herbal

Introduction

Erectile dysfunction (ED) affects 50% of men aged between 40 and 70 years and therefore is considered to be an important health problem.[1] As men age, several physiological or induced factors arise that contribute to ED, such as a decline in the testicular production of testosterone, vascular functionality, levels and responsiveness to vasoactive amines and neurotransmitters, diseases (for example, cardiovascular, hypertension, diabetes mellitus, chronic prostatitis) and certain drugs.[1, 2] A number of pharmacological agents are introduced to correct ED transiently, such as the orally consumed phosphodiesterase type 5 inhibitors, testosterone therapy, or vasoactive agents inserted intraurethrally or injected intracavernosally.[1-3]

Some natural products such as *Panax quinquelotius, Eurycoma longifolia and Epimedium grandiflorum* have the ability to act as an aphrodisiac and to help restore ED. The

medicinal activity of *Panax quinquelotius* (Ginseng) has improved penile rigidity, libido and patient satisfaction in men with ED,[4, 5] whereas using a *Eurycoma longifolia* extract (Tongkat Ali) and *Epimedium grandiflorum* (Horny Goat Weed) in animals increased sexual arousal, motivation and frequency of sexual activity.[6-10] Furthermore, there are some natural products that could play a role in improving circulation to the prostate and penis such as *Centella asiatica* (Gotu Kola) and flower pollen.[11-13] Therefore, it was our hypothesis that the development of a herbal combination of the above five plant extracts, called Etana,[14] could work on several age-induced causes of ED. On the basis of the mechanism of action of each component, this herbal combination could have an additive or synergistic effect to restore erectile function.

To introduce Etana as an enhancer of male erectile function, this study examines the efficacy of Etana in relation to each of its components, to its dose-response effect, in comparison to sildenafil as a known drug to restore erectile function and to different herbal combinations. In addition, acute and subacute toxicity studies of Etana were carried out to establish the safety of this herbal combination.

Materials and Methods

Herbal Extracts

Panax quinquelotius, Eurycoma longifolia and Epimedium grandiflorum extracts were purchased from Hongjiu Ginseng, the Active Ingredients Group and from the Chengdu Wagott Pharmaceutical Co., Chengdu, China, respective. *Centella asiatica* (Gotu Kola) and flower pollen extracts were purchased from Graminex, USA and Ennagram, France, respectively. Sildenafil citrate was obtained from JPM, Jordan.

Etana preparation and method of analysis

Etana is a mixture of Ginseng extract (100 mg), Tongkat Ali extracts (200 mg), Epimedium extract (50 mg), Gotu Kola extract (40 mg), and flower pollen extract (135 mg). The preparation was dissolved in distilled water and given to rats by oral gavage.

The method of analysis of Etana components is based on the HPLC method to assay a marker for each constituent (for example, icariin for *Epimedium grandiflorum* and malasiatic acid for *Centella asiatica*). The method is based on a solid stationary phase (C18 packed column), mobile phase, and separation by partition adsorption or ion exchange process. The gradient mixtures of acetonitrile: H_3PO_4 and the detection wavelengths were different for each component.

Animals

Male and female Wister rats (220-300 g) were obtained from the Yarmouk University animal house unit (Irbid, Jordan). The animals were housed at the animal facility in Petra University in a 12 h light or dark cycle at a constant temperature of 22° C. All animals were acclimatized for 10 days before the experiments with free access to a standard diet and drinking water. All animal experiments were carried out in compliance with relevant laws and institutional guidelines.

Sexual behavior and penile erection index

Each animal group consisted of 10 male rats weighing 200-300 g. Each test preparation was dissolved freshly in distilled water and doses were administered by oral gavage. Dosing of Etana was either as a single dose per day or as a triple dose per day, 3 h apart, to show any changes in the efficacy obtained from multiple administrations per day. Control animals were given the vehicle alone (distilled water). Rats were placed in glass cages, allowed free access to food and tap water and were observed for 3 h after each drug administration for penile erection, genital grooming and possible copulation mounting. The number of responding rats was recorded along with the number of sexual activity episodes (penile erection, genital

grooming or copulation mounting). Penile erection index (PEI) was calculated for each group by multiplying the percentage of active rats (responding rats) by the total number of activity episodes.[15-17]

Acute and subacute toxicity assessment

Acute toxicity for Etana was determined in rats (250-300 g) consisting of 10 rats per group (five males and five females). A single dose of 0, 7.5, 37.5, 75, 150, 225 and 300 mg kg^{-1} (that is, 1 x to 40 x of the human recommended daily dose based on 70 kg b.w.) was given by oral gavage to each animal per group. The animals were observed closely for any toxic or abnormal behavior in the first 2 h after dosing and were kept under further observation for 2 weeks.

A subacute toxicity study for 28 days was carried out according to ICH guidelines. A single dose of 0, 7.5, 15 and 75 mg kg^{-1} (that is, 1 x, 2 x, 10 x of the human recommended daily dose based on 70 kg b.w.) was given by oral gavage to each animal per group. Each test group consisted of five males and five females, and different sex animals were kept in separate cages to avoid pregnancy during the test period. Animals were monitored carefully and body weights were measured daily. At the end of 28 days, all animals were killed. Just before being killed, blood samples were taken from the jugular vein for a full blood and chemistry analysis. All internal organs were carefully removed, weighed and then fixed with 10% buffered formalin for histological examination.

Data analysis

All variables were analyzed using SPSS version 10 statistical package (SPSS Inc., Chicago, IL, USA) using different statistical tests. For sexual behavior and PEI analysis, Student's *t* test was carried out to compare the level of significance between groups. As for the toxicity study, statistics were generated for time interaction, gender effect and differences between each treatment group and the control group. The overall differences between the groups were analyzed using one-way ANOVA. In some cases, Turkey's post test was carried out after ANOVA to show the differences between selected groups. For all of the statistical comparisons, the level of significant difference was defined as $P<0.05$.

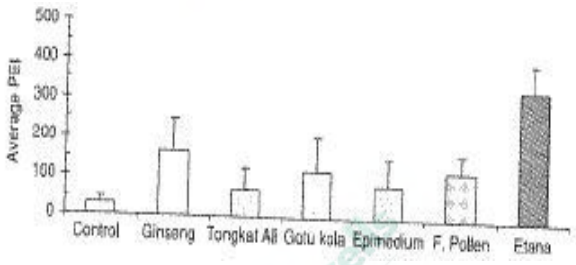

Figure 1. The PEI for different groups of rats administered either a single component of Etana, Etana or distilled water. The PEI was determined by multiplying the average scores reported during a 6-day treatment by the average percentage of responding rats. The rats were monitored for 3 h after a single administration. Each single group was administered the same amount (mg) as is present in Etana. Each bar point represents the mean of six experiments ± s.d.

Results

Etana versus each single component on male rat sexual behavior

The PEI after the administration of each single component of Etana in comparison with Etana is presented in Figure 1. In all treated animals, PEI increased significantly ($P<0.001$) when compared with control animals. In addition, Etana-treated rats showed significantly higher ($P<0.001$) PEI than did each single Etana component-treated rats (Figure 1). In addition, the PEI of Etana is higher than the sum of any two herbal components. Furthermore, the number of responding rats after a single administration of Etana was significantly higher ($P<0.001$) than each single Etana component-treated rat.

Dose response of Etana on male rat sexual behavior

The dose response of Etana showed a significantly higher ($P<0.001$) PEI at a dose of 7.5 mg kg^{-1} of Etana when compared with 2.5, 15 mg kg^{-1} and controls (Figure 2). In addition,

when Etana was administered thrice a day, 3 h apart to the same rats, the PEI was significantly higher ($P<0.001$) at 7.5 mg kg^{-1} dose when compared with that in the other doses and control, and the cumulative PEI did not change after the second or third dose to the same rats (Figure 2).

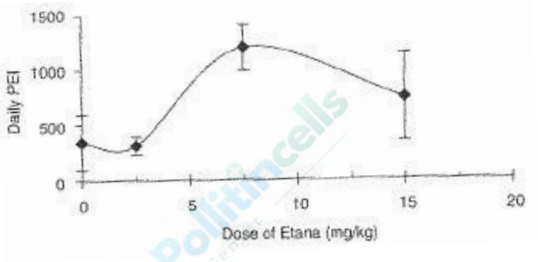

Figure 2. The PEI for different groups of rats administered different doses of Etana. Etana was administered thrice a day, and PEI was determined by multiplying the average scores reported during a 3-day treatment by the average percentage of responding rats. Each bar point represents the mean of three experiments ± s.d.

Etana versus Sildenafil on rat sexual behavior

In this set of experiments, the effect of Etana 7.5 mg kg^{-1} was compared with the effect of two therapeutic doses (0.36 and 0.71 mg kg^{-1}, based on 70 Kg b.w.) of sildenafil citrate. The PEI after Etana (7.5 mg kg^{-1}) administration as a single or triple dose per day was similar to that of 0.71 mg kg^{-1} of sildenafil and was significantly higher ($P<0.001$) than that of 0.36 mg kg^{-1} of sildenafil and the control group (Figure 3).

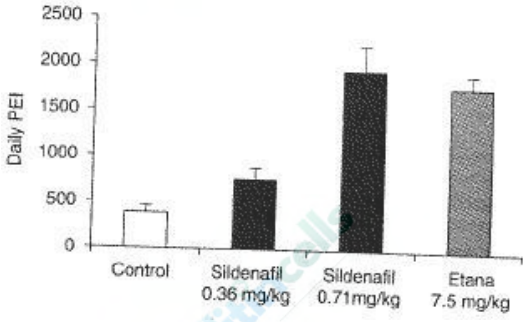

Figure 3. The PEI for different groups of rats administered two doses of sildenafil and Etana. Doses were administered thrice a day, 3 h apart, and PEI was determined by multiplying the average scores reported during a 3-day treatment by the average percentage of responding rats. Each bar point represents the mean of three experiments ± s.d.

Etana versus a different mixture of herbal components on male rat sexual behavior

The PEI after administration of different mixture components, group 1 (*Centella asiatica, Eurycoma longifolia, Epimedium grandiflorum, flower pollen extract and Gingko)* and group 2 (Ginseng, *Eurycoma longifolia* and *Epimedium grandiflorum*), is shown in Figure 4. The cumulative PEI and the percentage of responding rats after the administration of Etana thrice a day were significantly higher ($P>0.001$) than the PEI and percentage of responding rats of group1 and 2 (Figure 4). In group 1, Gingko was added instead of Ginseng because of its known function as an aphrodisiac. A single administration of Gingko (0.86 mg kg^{-1}) showed PEI and percentage of responding rats at 80 and 22%, respectively.

Acute and subacute toxicity of Etana

No deaths occurred after the administration of any of the single doses tested (7.5-300 mg kg^{-1}). After a 28-day administration of 7.5, 15 or 75 mg kg^{-1} (that is, 1 x, 2 x, 10 x of the effective dose), no deaths occurred, and the body weight did not show any significant changes in male or female rats. In addition, the weights of the internal organs did not show any changes after a 28-day administration of any of the doses tested.

The chemistry results after a 28-day administration of 1 x, 2 x, and 10 x dose of Etana showed no significant differences in triglycerides, ALT, AST, ALP, sodium, creatinine, calcium and phosphorus. However, a significant reduction in cholesterol, urea and potassium levels ($P<0.03$-0.001) was observed (Table 1). The reduction of cholesterol was dose dependent ($P<0.001$) as the percent reduction was 20, 26 and 34% for 7.5, 15 and 75 mg kg^{-1}, respectively, whereas the reduction of urea was dose dependent (15% for all doses; $P<0.03$) and the reduction of potassium was seen only at the

75 mg kg^{-1} dose (10%; P<0.015). On the other hand, the glucose level increased significantly only at 75 mg kg^{-1} dose (68%, P<0.025) (Table 1).

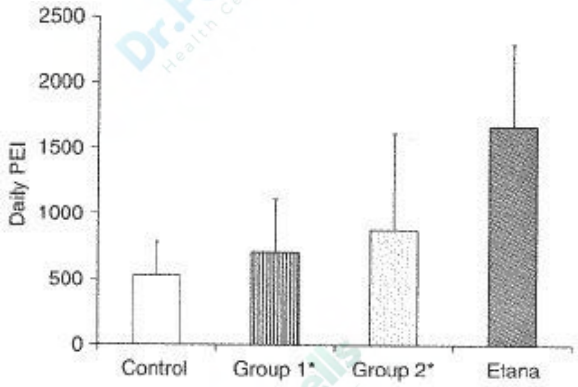

Figure 4. The PEI for different groups of rats administered different combinations: *Centella asiatica*, *Eurycoma longifolia, Epimedium grandiflorum,* flower pollen extract and Gingko for group 1: and Ginseng, *Eurycoma longifolia* and *Epimedium grandiflorum* for group 2 and Etana. Doses were administered thrice a day, 3 h apart, and PEI was determined by multiplying the average scores reported during a 3-day treatment by the average percentage of responding rats. Each single group was administered the same amount (mg) as is present in Etana. Each bar point represents the mean of three experiments ± s.d.

The hematological changes after a 28-day administration of 7.5, 15 and 75 mg kg^{-1} dose of Etana showed a significant increase in the percentage of lymphocytes, and a significant decrease in the percentage of neutrophils in peripheral blood at the doses of 15 and 75 mg kg^{-1} (P<0.05-0.001) (Table 1). However, the absolute number of the above cells in peripheral blood did not change because there was an apparent reduction in the total leukocytes count.

Discussion

This study describes a new and safe combination of herbal components that enhances erectile function in male rats. Most of the single constituents of Etana have been widely used for enhancing erectile function, and scientific evidence was reported to explain the mechanism of each component. The idea was to show the additive or synergistic effect of such combination. The results indicate that Etana showed a significantly higher percentage of responding rats and PEI. Furthermore, the Etana efficacy was dose dependent, showing higher activity at either single dose or triple dose of 7.5 mg kg^{-1} per day, and can be administered for a long period of time without any toxic effect.

To confirm our hypothesis with regard to the efficacy of Etana combination versus other possibilities, it was compared with two other combinations. The choice of the two other combinations was based on the known mechanism of each component. Group 1 was a mixture of *Centella asiatica, Eurycoma longifolia, Epimedium grandiflorum,* pollen extract and Gingko versus Ginseng, Eurycoma longifolia and *Epimedium grandiflorum* (group 2) and Etana (Ginseng, *Eurycoma longifolia*, *Epimedium grandiflorum*, *and Centella asiatica* and flower pollen). Group 1 components are similar to Etana except that it contains Gingko instead of Ginseng. Gingko has also been used for aphrodisiac effects but it has a different mechanism of action from Ginseng.[18] Group 2 does not contain flower pollen and *Centella asiatica.* On the basis of the above, one of the mechanisms of action of Etana as a herbal combination to enhance blood flow is consistent with the synergistic effects observed by combining the individual components.[11-13]

Table 1. The significant hematological and biochemical findings of rats treated with different doses of Etana for 28 days

Group	*Cholesterol (mg dl^{-1})*	*Urea (mg dl^{-1})*	*Potassium (mmol^{-1})*	*Glucose (mg dl^{-1})*	*Lymphocytes %*	*Neutrophils %*
Control	58 ± 2	37 ± 2	6.5 ± 0.2	55 ± 9	73 ± 3	13 ± 2
7.5 mg kg^{-1}	46 ± 5*	31 ± 2*	6.7 ± 0.2	56 ± 5	70 ± 3	14 ± 2
15 mg kg^{-1}	41 ± 3**	32 ± 2*	6.8 ± 0.3	75 ± 3	80 ± 2*	8 ± 1*
75 mg kg^{-1}	38 ± 2**	33 ± 1*	5.8 ± 0.2*	92 ± 12*	87 ± 2*	5 ± 1*

*P<0.05 when compared with the control group, **P<0.001 when compared with the control group.

On the basis of the published scientific evidence of each Etana component, the mechanism of action of Etana can be fourfold. First, it has been shown that ginsenosides, which are extracted from Panax ginseng, increased the plasma levels of FSH, LH, testosterone (total and free forms) and spermatozoa concentration and motility.[4] This suggests that ginsenosides act on the hypothalamus and or pituitary to increase plasma FSH and LH, thus activating testes to increase testosterone levels and spermatozoa formation. [4, 19] Second, it was found that *Eurycoma longifolia* enhanced the testosterone effect by increasing the sexual performance of inexperienced castrated male rats. [6] Third, it has been shown that ginsenosides and icariin, isolated from *Epimedium grandiflorum*, promoted the release of nitric oxide (NO)from corpus cavernosum.[10, 20] The release of NO induces the relaxation of the smooth muscle and thus enhances erection. In addition, ginsenosides and icariin were found to increase intracavernosal pressure.[10, 20] Furthermore, icariin was found to be a cGMP-specific phosphodiesterase 5 inhibitor *in vitro*,[24] but not *in vivo*, after oral dosing for 4 weeks.[10] In this study, however, the dose response of Etana showed a bell-shaped curve (Figure 2), suggesting a phosphodiesterase inhibition. Fourth, the addition of flower pollen extract and *Centella asiatica* improves blood circulation to the prostate and penis, thereby enhancing the level of the other components (or their effects) of Etana to reach the genital tract.[11-13] Furthermore, it is known that one of the major problems that could result in ED is chronic prostatitis.[2, 13] Both *Centella asiatica* (Gotu Kola) and flower pollen have antioxidative activities that are important to reduce male infertility and help in managing chronic prostatitis.[11, 13]

In addition to enhancing erectile function, Etana has other benefits. It lowered the serum cholesterol level after 28 days of oral dosing in a dose-dependent manner. This cholesterol-lowering effect is mainly due to Panax ginseng and flower pollen.[21-23]

It has been shown that Panax ginseng lowers cholesterol and triglyceride levels by activating lipoprotein lipase in hyperlipidemic rats.[22] In this study, however, the rats were normal and 28 days of Etana administration did not cause any significant change in the triglyceride levels.

This paper describes a new combination of herbal extracts that enhances erectile function and is safe after a long day of use. In addition, this herbal combination could also help in reducing the serum cholesterol level and in managing chronic inflammation of the prostate.[11-13] Clinical studies are warranted for evaluating Etana's significance in ED and in men with chronic prostatitis.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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MUSCLE SUPPORT:

GRAMINEX Flower Pollen Extract

Effect of Nutritional Substances* on Work Capacity during Stay in a Subtropical Climate

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Through a recently performed experiment involving 90 Polish soldiers – divided into three groups during a stay in a subtropical climate – it was possible to demonstrate deterioration in physical and mental capacity during the period of adaptation. Administration of preparations containing pollen extracts and amino acids significantly improved physical performance, for example, in long-distance running, long jumping, the formation of lactate after exercise on a bicycle ergonometre, as well as concentration and subjective well-being.

As yet, no explanation can be offered for the effects observed.

In 1978, some Polish soldiers participated in the peacekeeping forces stationed by the United Nations at the Suez Canal following the Egyptian-Israeli conflict.

The region has a subtropical climate, with an average temperature approaching 30°C during the six months of summer. Past experience has shown that such temperatures normally affect people from more temperate climates. The result is that work capacity and physical performance are impaired over a fairly long period of adaptation. In addition, concentration and subjective well-being are adversely affected.

In order firstly to survey the extent of such changes and secondly to establish whether the changes could be affected by the administration of pollen extract and amino acids, 90 soldiers were subjected to a thorough examination before and during a 5-month stay in this region. The chosen method of therapy was partly based on an experiment by Jethon and others on weightlifters, in which these preparations were shown to affect significantly the weightlifters' physical performance.

Test Subjects:

90 Polish soldiers, with special training as drivers, were assigned at random to 1 of 3 companies to serve at the Suez Canal. Ages varied between 20 and 33 years, average weight was 66 kg and average height was 171 cm. All were in good health, and were mentally and physically fit.

Procedure:

Soldiers in two of the three platoons were given nutritional preparations, while the third served as a control group. One of the first two groups was given only pollen extract (Pollitab Sport – 4 tablets – daily, at mealtimes). The second group also received extra amino acids ("Stark-protein", 1/4 g daily), also at mealtimes. All groups had identical diets, duties and training programs.

*Pollen extracts and amino acids.

The soldiers' physical performance was analyzed before their departure from Poland, then after 1, 2, 3, 4, 6, 8, 12, 16, and 20 weeks in the subtropical climate.

A number of measures of performance were used to evaluate physical performance, including running for various periods of time, press-ups, long-jumping and increases in the blood's level of lactic acid following standard exercise on a bicycle ergonometre. Physical performance was measured at the same times of day, including by the Bourdon test of speed and mental efficiency, the Wiersma test of concentration, and subjective assessments of well-being on an analogue 7-point scale.

The results obtained were dealt with using normal statistical procedures and the Student t-test.

Results:

Full results from the experiment were published in a doctoral dissertation (Dabrowski, 1980). The main positive findings are summarized below.

In practically all indications obtained of physical and mental performance, there was a significant, distinct deterioration during the first two to three weeks in the specific climate. This corresponds fully to findings from previous experiments (Falkiewics, 1966, 1971 and Galubinski, 1979). A gradual recovery was then observed during the 5-month long stay, but the degree of recovery differed considerably from individual to individual. In the majority of individuals and variable values, the recovery never matched the original values.

The interesting result of this experiment was the consistent and typical difference obtained for most variables throughout the three groups. Recovery in the control group was slowest and the least complete, while recovery in the groups given the nutritive was clearly fuller and more rapid. The recovery in performance was greatest in the group given the combination of pollen extract and amino acids. A comparison between the latter group and the control group revealed a significant difference ($p<0.01$) for 1,000 metres running, long jumping, lactate increase after exercise and tests of concentration and subjective well-being. (Figures 1-5).

Figure 1

1,000 metres

Min. –sec.

Pollen + amino acids
Pollen
Control group

Weeks

Figure 2

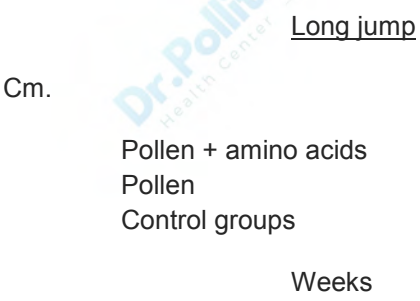


Figure 3

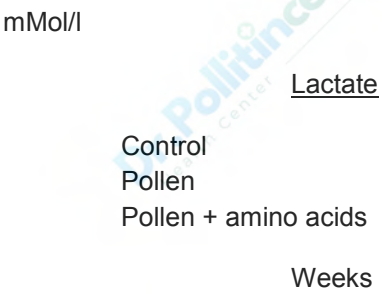


Figure 4

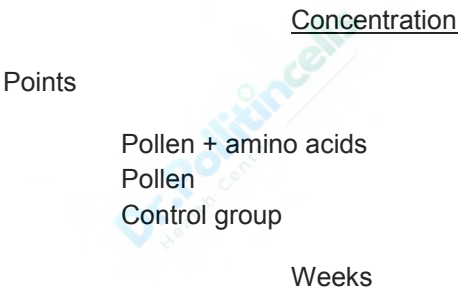
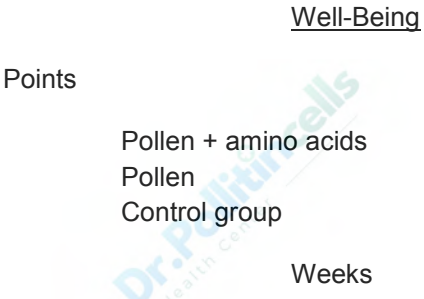


Figure 5



Discussion:

This experiment clearly confirms previous findings – for example, by Jethon and others –that physical performance is improved by the administration of certain nutritional susbstances. It may also be said that the changes observed are remarkably substantial and it would be desirable for the results to be explained, for example, by metabolic change (lactate formation during exercise, enzyme effect, etc.). However, such an explanation cannot easily be offered without further research. Although amino acids make a valuable contribution to physical build-up and enzyme synthesis, the quantity of protein administered is too little, in comparison to a normal daily protein intake (70 g), to have any significant impact on the nitrogen balance. It is possible that administering a balanced intake of all the amino acids in this way could be beneficial, in comparison with intake via a normal diet. However, since both milk and meat formed part of the diet, this hypothesis does not provide an adequate explanation either. Similarly, it is hard to account for the effect of the pollen extracts. In previous experiments, it has been observed that these extracts had a “performance-raising” and roborant effect (Dubrisay, 1978). However, the problem here is that an extract knowledge of all the active ingredients in pollen extracts is not available. A number of different growth steroids with growth-stimulating effects in plants could be the active agents.

Nevertheless, considerably more research is needed in this area if these strong, but difficult to explain, correlations between nutritional preparation and performance are to be accounted for.

Summary:

Through a recently performed experiment involving 90 Polish soldiers – divided into three groups during a stay in a subtropical climate – it was possible to demonstrate a deterioration in physical and mental capacity during the period of adaptation. Administration of preparations containing pollen extracts and amino acids significantly improved physical performance, for example, in long-distance running, long jumping, the formation of lactate after exercise on a bicycle ergonometer, as well as concentration and subjective well-being.

As yet, no explanation can be offered for the effects.

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MUSCLE SUPPORT:

GRAMINEX Flower Pollen Extract

Effects of Pollen Extract upon Adolescent Swimmers

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Abstract

Many competitive sportsmen in this country regularly use pollen extract as a dietary supplement in the belief that it can lead to an improvement in performance. We have investigated the effects of a six-week course of pollen extract administration on a variety of physiological parameters in a group (n=20) of adolescent swimmers. At the time of the study, all subjects were training on a daily basis. During the course of the study, maximum oxygen uptake increased in both the treatment group and the placebo group, no differences between the response of the two groups being observed. Vital capacity showed a significant increase in the treatment group, but not in the placebo group. The results indicate that no positive benefit was obtained from the use of pollen supplementation. However, the number of training days missed due to upper respiratory tract infections was much less in the pollen treatment group (4 days) than in the placebo group (27 days). In a study of longer duration, this difference could lead to an improved performance by the pollen treatment group due to fewer interruptions to training.

Keywords: Performance, Swimming, Pollen, Diet.

Introduction

The sporting situation is an intensely competitive one in which athletes will search for any improvement in performance, however small it may be. Accordingly, a wide range of ergogenic aids in the form of dietary supplements is extensively employed in this field. One of the more recent innovations is the use of a pollen extract which has been claimed to produce improvements in athletic performance. This product has been marketed by A. B. Cernelle as “Pollitabs,” containing pollen extract as well as vitamins B, C and E. The two main beneficial effects of this product have been claimed to be an increased resistance to respiratory tract infection (Lindahl, 1978; Mark-Vendel, 1978) and an effect on protein synthesis (Dubrisav, 1972). A large number of the studies carried out in this area have been rather poorly controlled, and it was the aim of the present study to determine whether or not administration of a pollen extract could influence performance.

Methods

Twenty young competitive swimmers were used as subjects, comprising 16 males and 4 females. Mean age of the subjects was 15.7 years (range 11.5 to 20 years). All subjects were training on a daily basis, and all had been training for some months prior to the beginning of the test period. Subjects were divided into two groups, each containing 8 males and 2 females, the selection being random otherwise. A series of tests as detailed below, was then performed on each subject. These tests were repeated after a six-week period during which subjects ingested either Pollitabs or placebo (cod-liver oil capsules). Treatment administration was performed on a double-blind basis in order to minimize any subjective bias. Differences

between groups were assessed by the Student's t-test, and differences within each group as a result of treatment were assessed by a paired t-test.

Height and weight were recorded, in addition to percentage body fat according to the method of Durnin and Rahaman (1967). Right and left hand grip strength measurements were made using a grip dynamometer (Takentiki Vogyo, Japan). Quadriceps isometric strength (MVC) and endurance time at 50% of MVC were measured using an isometric chair constructed after the manner of Thorstensson (1976).

Maximum oxygen uptake (VO_2 max) was assessed by using stepwise increases in workload on a friction braked Monark bicycle ergometer. The attainment of VO_2 max was established according to the levelling off criterion of Astrand and Saltin (1961). Respiratory variables were assessed on a P.K. Morgan automatic gas analysis system comprising a Fleisch pneumotachograph, paramagnetic O_2 analyzer and infra-red CO_2 analyzer.

Vital capacity (VC) and forced expiratory volume (FEV_1) was obtained using a Vitalograph spirometer.

Haemoglobin (Hb) concentration was estimated by conversion to cyanmethemoglobin using Drabkin's reagent (BDH); haematocrit was obtained using a Hawksley micro-haemoatocrit system.

Results

Results are presented in Table 1.

Anthropometric Measurements

Body weight and height increased in both groups during the test periods ($p < 0.01$). There were no differences between groups either before or after the test period. There were no differences in body fat content between the groups, and no change took place in either group.

Strength and Endurance Tests

No significant changes took place in right hand grip strength, isometric leg strength and isometric endurance time, with no differences between the groups. However left hand grip strength showed significant improvement in both groups ($p < 0.05$) in both groups). No

TABLE I

Comparison of Pollitabs and placebo groups. Values are means ± SEM. The right hand column shows the differences between the changes observed in the two groups. A full explanation of the tests employed is given in the text.

	Test Group			Control Group			
	Pre	Post	Difference	Pre	Post	Difference	Test v Control
Height (cm)	167.1 ± 2.2	167.8 ± 2.2	+0.7	165.2 ± 3.7	165.8 ± 3.7	+0.6	+0.1
Weight (kg)	56.2 ± 3.3	58.3 ± 3.3	+2.1	58.2 ± 3.1	60.0 ± 2.8	+1.8	+0.3
Body fat (%)	15.0 ± 1.2	15.4 ± 1.1	+0.4	18.0 ± 2.0	18.5 ± 2.0	+0.5	−0.1
Grip strength (R, kg)	38.8 ± 4.0	39.6 ± 4.0	+0.8	37.1 ± 2.4	36.7 ± 2.1	−0.4	+1.2
Grip strength (L, kg)	35.3 ± 3.6	36.6 ± 3.6	+1.3	34.1 ± 2.1	35.8 ± 2.2	+1.7	−0.4
Leg strength (kg)	40.7 ± 3.7	44.8 ± 3.4	+4.1	42.8 ± 2.3	47.0 ± 4.0	+4.2	−0.1
Endurance (sec)	81 ± 6	72 ± 6	−9	73 ± 7	72 ± 8	−1	−8
VC (l)	4.83 ± 0.33	4.99 ± 0.30	+0.16	4.88 ± 0.38	4.96 ± 0.38	+0.08	+0.08
FEV (%)	86.8 ± 2.0	85.9 ± 1.6	−0.9	81.0 ± 1.0	82.0 ± 1.1	+1.0	−1.9
VO_2 max (l) min	3.05 ± 1.8	3.30 ± 2.0	+0.25	3.05 ± 0.25	3.32 ± 0.21	+0.27	−0.02
VO_2 max (ml/kg/min)	54.4 ± 2.1	56.5 ± 1.4	+2.1	52.8 ± 2.2	55.1 ± 1.8	+2.5	−0.4
Hb (g%)	16.8 ± 0.3	16.0 ± 0.3	−0.8	16.7 ± 0.4	15.6 ± 0.5	−1.1	+0.3
Hct (%)	43.5 ± 0.9	42.3 ± 0.9	−1.2	42.0 ± 1.4	41.1 ± 1.3	−0.9	−0.3

differences, however, were found to exist between the groups.

Blood Measurements

A significant decrease ($p < 0.05$) in blood haemoglobin concentration took place during the trial period with mean (± SD) reductions of 1.05 ± 0.91 and 0.61 ± 0.67 g/100 mls for placebo group and Pollitabs group respectively.

The haematocrit (percentage) was found to decrease in the Pollitabs group significantly ($p < 0.05$); however there were no differences between the two groups.

Aerobic Capacity

The VO_2 max, expressed in 1/min, showed a significant improvement in both the Pollitabs and placebo group ($p < .05$), with mean (±SD) increases of 0.27 ± 0.09 and 0.24 ± 0.09 l/min respectively. If allowance is made for the increase in body weight which occurred during the test period, this increase in VO_2 max, expressed in ml/kg/min does not assume statistical significance.

Respiratory Parameters

Vital capacity increased considerably in the group taking Pollitabs ($p < 0.05$); a small increase was also observed in the control group, but this did not attain statistical significance. No significant difference was found between the groups.

Force expiratory volume in 1 second was not found to change in either group nor was any significant difference found between the groups.

Discussion

The subjects used for the present study were healthy adolescents differing from the normal population only in that they were all engaged on a strenuous programme of physical training. They may thus be considered to represent the group at which the beneficial effects of pollen supplementation are aimed.

If the body weight and height of the these subjects are compared with non-athletic children of comparable age, almost all were heavier and taller than average (Bayer and Bayler, 1976); this is in agreement with results obtained by Eriksson et al (1977) for a comparable population of swimmers. The results did not indicate the administration of Pollitabs had any effect on body weight, height or body fat content. It would not, however, be expected that any such effects would become apparent within the time scale of this experiment.

The normal training programme undertaken by the subjects included two weight training sessions weekly, with the aim of increasing muscular strength. In spite of this, there were no significant increases in either group recorded for right-hand grip strength or for quadriceps strength. In contrast to this finding the left-hand grip strength showed comparable increases in both groups. This may be explained by the fact that the left-hand strength is generally weaker than the right; any bilateral training carried out would thus represent a greater stimulus to the left side and consequently produce a greater improvement in performance.

The significance of the changes in vital capacity is not immediately clear. The results show an increase in VC in the Pollitabs group but not in the placebo group. This change, however, is not sufficiently large to cause a significant difference to exist between the two groups. The values obtained for all subjects in the present study are higher than those of normal adolescents (Engstrom et al, 1962). This finding is in agreement with other results obtained from swimmers (Andrews et al, 1972; Eriksson et al, 1977) and is also in agreement with the suggestion that a large VC is required for success in competitive swimming (Astrand et al, 1963).

A higher correlation has also been shown to exist between VO_2 max recorded during work on a bicycle ergometer and swimming performance (Astrand et al, 1963). Although both groups recorded a higher value for VO_2 max following

the test period there was no difference between the two groups, and the difference can therefore be ascribed to the effects of the training regimen.

The changes in hematological variables (Hb and Hct) which were recorded would appear to be of little consequence and probably reflect the haemodilution which normally accompanies a period of physical training.

The results would appear to indicate that there is no beneficial effect to be obtained by administration of pollen extract to swimmers. Before this conclusion can be stated with any certainty however, two points must be born in mind. This first of these is that the present test lasted only six weeks; by comparison with the time scale which most training programs are conducted, this is an extremely short space of time and may not be of sufficient duration to produce a measurable effect. Secondly, it was noted that the placebo group, during the 6-week experimental period, missed a total of 27 days of training through illness while the Pollitabs group missed 4 days in total. All days missed in both groups were the results of upper respiratory tract infections. Because of the small numbers involved, these data are not readily amenable to statistical evaluation. They do, however, suggest that the swimmers taking Pollitabs might expect to miss only 1 day in 105 due to upper respiratory tract infection; this compares extremely favorably with the placebo group who might expect to miss 1 day in 16. Such a difference might be expected to have important consequences for the athlete whose performance is dependent on the ability to engage in consistent physical training.

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OTHER SUPPORT:

GRAMINEX Flower Pollen Extract

Demonstration of superoxide dismutase enzymes in extracts of pollen and anther of *Zea mays* and in two related products, Baxtin ® and Polbax®

PER CHRISTER ODÉN, GÖSTA KARLSSON and ROLAND EINARSSON

Odén, P.C., Karlsson, G. & Einarsson, R. 1992. Demonstration of superoxide dismutase enzymes in extracts of pollen and anther of *Zea mays* and in two related products, Baxtin® and Polbax®. – Grana 31: 76-80, 1992. Odense, February 1992. ISSN 0017-3134.

Superoxid dismutase enzymes were isolated and identified in an aqueous extract of pollen and anthers of *Zea mays* and in two commercial products, Baxtin® and Polbax®, derived from the same kind of source material. Prior to analysis the samples were purified by adding soluble poly-N-vinylpyrrolidon and precipitated with ammonium sulphate.

Gel filtration chromatography using a Superose 12 HR column gave well-resolved and similar elution patterns for the maize extract, Baxtin and Polbax samples. Fractions exhibiting superoxide dismutase activity, determined by direct KO_2 assay, were combined, dialysed and evaporated to dryness. The molecular weight of these fractions was approximately 30000 d. These fractions were also analyzed by native polyacrylamide gel electrophoresis and stained for superoxide dismutase enzyme activity using nitro blue tetrazolium. The major region of superoxide dismutase enzyme activity was inhibited by addition of cyanide and hydrogen peroxide indicating the presence of a copper, zinc superoxide dismutase. Another minor region of enzyme activity, migrating as standard manganese superoxide dismutase and not inhibited by cyanide or hydrogen peroxide, was also detected.

The results clearly demonstrated the occurrence of both copper, zinc- and manganese superoxide dismutase enzymes in extracts of pollen and anthers of *Zea mays* and also in the two related commercial products, Baxtin and Polbax.

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Active oxygen in biological systems has been thoroughly studied and frequently reviewed (Elstner 1987, Salin 1987, Alscher & Amthor 1988, Leshem 1988, Winston 1990). The harmful effect of active oxygen can be reduced by keeping the O_2-concentration low within the cell through oxidative metabolism or oxygnease reactions. Other possibilities are to keep substrates that produce toxic interme diates separated from each other or by scavenging systems, antioxidants, that reduce the concentrations of active oxygen species. The antioxidants can either be fat-soluble, e.g., carotenoids and α-tocopherol, water-soluble, e.g., glutathione and ascorbic acid, or enzymatic

compounds, e.g., glutathione reductase and superoxide dismutase (SOD).

SOD is a metalloenzyme or actually a group of metalloenzymes that catalyzes the disproportionation of O_2^-. to yield molecular O_2 and H_2O_2 (Weselake et al. 1986). SOD protects cells against oxygen toxicity by scavenging the superoxide radical and SOD is therefore a key component in the free radical detoxification process. SOD-enzymes have been extensively studied during the last ten years and are now considered present in all aerobic organisms (Rabinowitch & Fridovich 1983, Fridovich 1986, Asada 1988. Monk et al. 1989, Hassan & Scandalios 1990). Three types of SODs have been found in plants, classified according to the metal at the catalytic centre: copper and zinc SOD (CuZn SOD), a manganese-containing homo dimer (Mn SOD) and an iron-containing homo dimer (Fe SOD) (Fridovich 1986). These three types can be distinguished by their sensitivity to inhibitors, CuZn SOD is inhibited by cyanide and H_2O_2 and the Fe SOD is inhibited by H_2O_2, whereas the Mn SOD is insensitive to both inhibitors. Most CyZn SODs isolated so far, with few exceptions, are homodimers with a molecular weight of approximately 32.000 (Fridovich 1986). The most abundant SODs in plants are the CuZn SODs, which are found mainly in the cytosol and chloroplasts. Mn SOD is usually localized to the mitochondrial matrix in higher plants (Asada 1988) but has also been isolated from chloroplast thylakoids (Hayakawa et al. 1985). In addition it has been detected in the glyoxysomes (Del Rio et al. 1983, Sandalio & Del Rio 1987).

The development of reproductive structures, e.g., pollen and spores, and the pollination and critical phases in the life cycle of plants. As good protection against the detrimental effects of free radicals is therefore necessary to avoid damage to the genetic information. Recently, Acvedo & Scandalios (1990) reported on the expression of SOD genes in mature pollen of maize.

The purpose of the present investigation was to demonstrate the presence of SOD enzymes in three different extracts of pollen and anthers of maize (*Zea mays*); freshly prepared maize extract, Baxtin and Polbax.

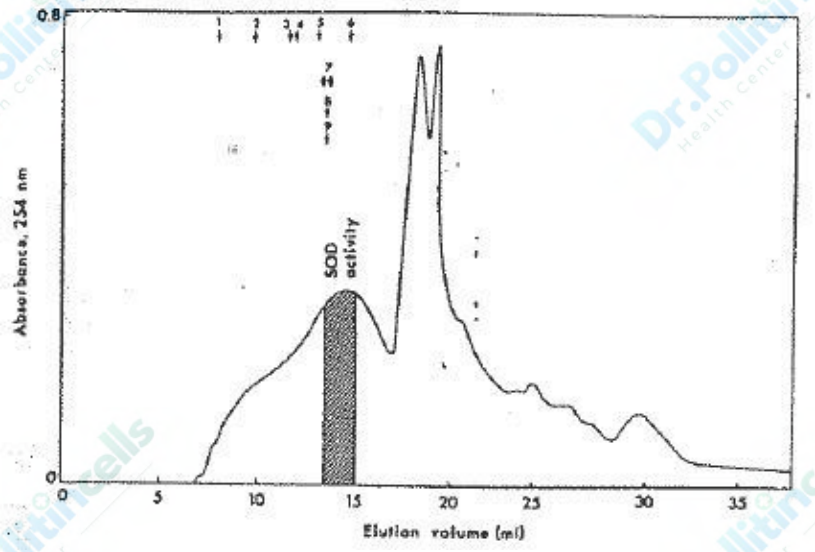

Fig. 1. Superose 12 gel filtration pattern of the maize pollen and anthers extract. The marked area contained SOD activity. Arrows indicate retention volumes of the molecular weight markers and the standard SOD enzymes; thyroglobulin (1), ferritin (2), catalase (3), aldolase (4), ovalbumin (5), chymo-trypsinogen A (6), CuZn SOD (7), Mn SOD (8) and Fe SOD (9).

MATERIALS AND METHODS

Preparation and purification of maize extract, Baxtin-solution and dissolved Polbax tablets

Fresh pollen and anthers of *Zea mays* (ratio 10:1 w/w) were incubated for 18 h in 200 ml of 50 mM TRIS-HCl, pH 7.5, containing 5 mM $MgCl_2$, 10 mM 2-mercapto-ethanol, 0.4 mM ascorbate, 2 mM EDTA and 4% poly-N-Vinylpyrrolidone 360 (PVP) (w/w). After incubation the sample was homogenized in an ice-bath with an Ultra-Turrax at maximum speed for 5 min. The tissue debris was removed by filtering and the sample was carefully brought to 20% saturation with saturated ammonium sulphate solution, stirred for 30 min and the precipitate was removed by centriguation at 5000 X g for 30 min. The supernatant was then brough to 90% saturation with addition of solid ammonium sulphate and stirred for 1 h, The precipitate was collected by contrifugation at 5000 X g for 30 min, and dissolved in 2.5 ml of 10 mM TRIS-HCl, pH 7.0. The SOD activity in this extract was tested by the direct KO_2 assay as described below and thereafter isolated using high-performance liquid chromatography equipped with a Superose TM 12 HR 10/30 column (highly cross-linked agarose matrix, Pharmacia LKB Biotechnology, Uppsala, Sweden). The sample was injected off-column via an injector with a 200 µl loop and the column was eluted with 5 mM phosphate buffer containing 0,15 m NaCl at a flow rate of 0.75 ml per min. The absorbance of the eluate was monitored at 254 nm. Forty fractions of 0.75 ml each were collected and the fractions were tested for SOD activity using the direct KO_2 assay.

For calibration of the Superose gel filtration column the elution volumes of the following molecular weight markers was determined; thyroglubulin 669.000 d, ferritin 440.000 d. catalase 232.000 d, aldolase 158.000 d, ovalbumin 43.000 d, chymotrypsinogen A 25.000 d (Pharmacia LKB Biotechnology) and standard SOD enzymes; CuZn SOD from Horseradish, Mn SOD from *E. coli* and Fe SOD from *E. coli* (Sigma Chemical Company, St. Louis, MO, USA).

Baxtin is a commercially available extract solution of fresh and well-controlled pollen and anthers of *Zea mays* (ratio 10:1 w/w) manufactured by Allergon AB, Välinge, Sweden. Ten ml of Baxtin solution (batch no 97808101) was carefully added in drops while stirring to 190 ml of 50 mM TRIS-Hcl, pH 7.5, containing 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.4 mM ascorbate, 2 mM EDTA and 4% PVP 360 (w/w). The Baxtin solution was then treated as described above for the freshly prepared maize extract of pollen and anthers.

Polbax (Allergon AB) is a commercially available product produced from a specified amount of grass pollen extract (Pollixin) and Baxtin. Polbax (210 tablets, batch no Q 186218) were ground to a fine powder and dissolved im 200 ml of 50 mM TRIS-HCl, pH 7.5, containing 5 mM EDTA and 4% PVP 360 (w/w). The Polbax solution was thereafter treated in an analogoua manner to the previously described maize extract.

Determination of SOD activity by the direct KO_2 assay

The SOD enzyme activity in the sample was measured by analyzing the disproprotionation of O_2^-. according to Marklund (1982). The analyzed samples were added in volumes of 20-40 µl to a 1 cm quartz cuvette containing 3 ml of 50 mM 2-amino-2-methyl-1 propanol (AMP)-HCl plus 0.2 mM diethylenetriamine penta acetic acid (DTPA), pH 9.5 and 5 µl 30 µM bovine liver catalase. KO_2 was dissolved in ice cold 50 mM NaOH plus 0.5 mM DTPA and 15 µl of this solution was added as substrate to the reaction mixture. The decay of the added O_2^-. was then measured continuously until the baseline had stabilized. KO_2 was added several times to confirm the enzymatic nature of the disproportionation reaction of the analyzed samples. The sensitivity of the disproportionation of O_2^-. to CN^- was also

monitored by adding 30 µl of 0.3 M NaCN to the reaction mixture.

Polyacrylamide gel electrophoresis (PAGE) and NBT staining for SOD activity

Native PAGE was performed at 15° C using PhastSystem and PhastGel homogenous 20 (Pharmacia LKB Biotechnolygy). Pooled fractions containing SOD activity form the gelfiltration chromatography were dialysed for 24 h against 4 l of 5 mM TRIS-HCl buffer pH 7.0 and finally evaporated to dryness. The samples were thereafter dissolved in 10 mM TRIS-HCl buffer, pH 7.0 and applied om native PAGE. The standard SOD enzymes were run in parallel.

The gels were immediately stained for SOD enzyme activity according to Beauchamp & Fridovich (1971) after finished electrophoresis. The gels were first soaked in darkness in 2.45 mM nitro blue tetrazolium (NBT) for 20 min and then soaked in 28 mM tetramethyl ethylene diamine plus 28 µM riboflavin for 15 min. All reagents were dissolved in 36 mM phosphate buffer pH 7.8. The effect of inhibitors in the activity was studied by adding 10 mM NaCN and 10 mM H_2O_2, respectively, to the solutions. After staining in darkness the gels were carefully washed with water and then treated with light. Regions with SOD enzyme activity were then visualized as lighter parts on the darkblue gel.

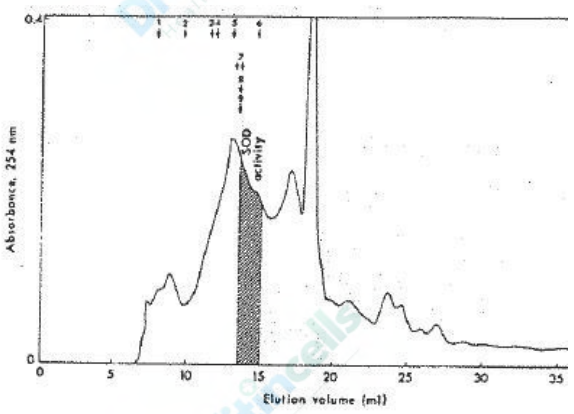

Fig. 2. Disproportionation of O_2^- by a fraction of the pollen and anthers extract measured spectrophotometrically at 250 nm after various additions of KO_2 and the effect of CN^- on the disproportionation of O_2^-.

RESULTS AND DISCUSSION

SOD enzyme activity was measured by the direct KO_2 assay in the crude extract of pollen and anthers of *Zea mays,* Baxtin and dissolved Polbax tablets. Due to analytical disturbances in the samples, however, it was not possible to obtain well-resolved protein bands with defined SOD enzyme activity on native PAGE gels after NBT staining.

Therefore the samples had to be purified to localize the active SOD enzymes in the electrophoretic patterns. The first step in the purification sequence was to absorb interfering compounds, mainly phenols, to soluble PVP. The second step was to apply the maize extract on a Superose 12 HR 10/30 column for further purification by gel filtration chromatography. The gel filtration elution pattern of the pollen and anther extract is shown in Fig. 1. SOD enzyme activity was detected by the direct KO_2 assay infractions eluting between 13.5 and 15 ml. These active fractions were pooled for further analysis. Fig. 2. shows the O_2^- disproportionation activity of the fractions eluted at 14.25-15.00 ml versus time after several additions of KO_2. The repeatability of the activity pattern after KO_2 addition for this maize fraction indicates that

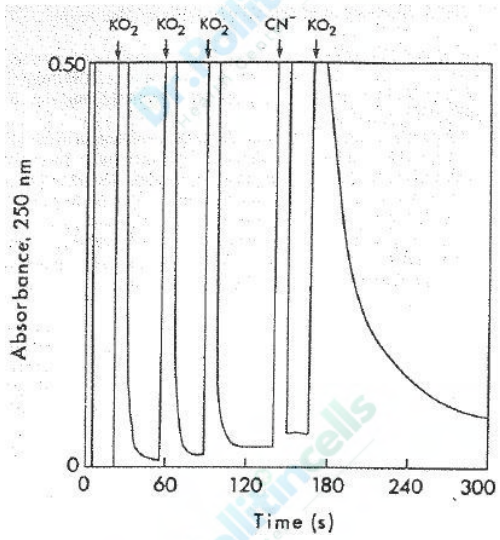

Fig. 3. Superose 12 gel filtration pattern of the Baxtin sample. Experimental conditions as in Fig. 1.

the molecule(s) responsible for the disproportionation reaction are not consumed and thus constitute an enzyme molecule. Furthermore the effect of CN^-, a Cu ZnSOD enzyme inhibitor, on the O_2^- -disproportionating activity of the maize fraction is also shown in Fig. 2. The maizederived pollen and anther fraction exhibits SOD enzyme activity and the main part of this activity is inhibited by the addition of CN^-. The cyanide sensitivity of the maize fraction confirms that the enzyme is copper and zinc-containing SOD (Fridovich 1986).

Fig. 1 also presents data about elution volumes of the molecular weight markers. The identified SOD enzymes at 2500 d and 43000 d and further the standard SOD enzymes chromatographed in parallel with the maize extract also elutes in this molecular weight region. A linear regression of the logarithmic molecular weights versus retention volumes resulted in a coefficient of determination of 0.97 and an approximate mean molecular weight of the putative SOD enzyme of 28.600.

The gel filtration elution profiles of the purified Baxtin and Polbax samples are shown in Figs. 3 and 4. The Superose elution patterns of Baxtin (Fig. 3) and the freshly prepared maize extract (Fig. 1) are similar, while the pattern for Polbax differs in the low molecular weight region (Fig. 4), most likely due to the presence of grass pollen components (e.g., Pollixin). However the elution volume of the fractions containing SOD enzyme activity is located in the same region. Both Baxtin and Polbax samples exhibited maximum SOD enzyme activity in the fractions eluting between 13.5 ml and 15 ml. The main part of the SOD enzyme activity was also inhibited by the addition of 10 mM CN^- to the reaction medium when performing the direct KO_2 assay, confirming the presence of CuZnSOD in Baxtin and Polbax.

The homogeneity of the SOD enzyme activity in the pooled fraction from Superose gelfiltration

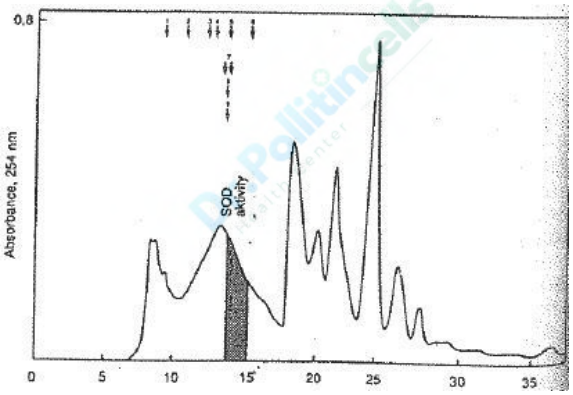

Fig. 4. Superose 12 gel filtration pattern of the Polbax sample. Experimental conditions as in Fig. 1.

was analyzed by PAGE. The polyacrylamide gel used allow complex mixtures of proteins to be separated in the molecular weight range 2000-150000 d. Fig. 5 illustrates three native PAGE gel patterns with standard CuZn SOD, FeSOD. MnSOD and the pooled fractions (13.5-15.0 ml) of the freshly prepared maize extract, Baxtin and Polbax samples in the absence and presence of SOD enzyme inhibitors during the staining procedure. The SOD enzyme activity in the various maize samples applied on the gel was developed by using the NBT reagent.

Fig. 5A demonstrates distinct SOD enzyme activity for all three analyzed maize samples (lane b, c, d) and further the activity is localised to defined regions on the gel. The enzyme activity of the SOD standards run in parallel is easily localised in the PAGE gel pattern. The enzyme activity of standard CuZn SOD and MnSOD partly overlap the SOD enzyme activity of the maize samples. Fig. 5B shows a PAGE analysis where the gel was treated with 10 mM CN^- and Fig. 5C with 10 mM H_2O_2 during the staining procedure. Inactivation of the enzyme activity (lane b, c, d) in the presence of CN^- confirmed that the SOD activity was due to CuZnSOD (Fig. 5B). The region located closer to the front was clearly inhibited by the addition of CN^- and H_2O_2 as was also the standard CuZn SOD (Fig. 5B and 5C). On the contrary the region close to the application point on the

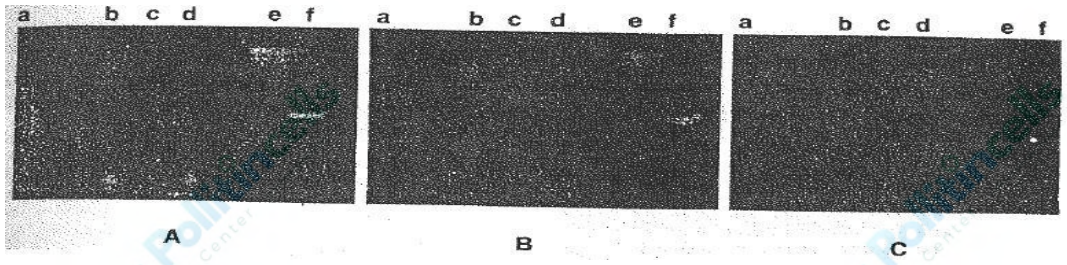

Fig. 5. A-C. Native PAGE gels stained for SOD enzyme activity with NBT after analysis of standard CuZn SOD (500 ng) (a), pollen and anthers extract (b), Baxtin sample (c), Polbax sample (d), standard Mn SOD (250 ng) (e) and standard Fe SOD (250 ng) (f). Polyacrylamide gels; (A) without inhibitor (B) in the presence of 10 nM CN- during staining and (C) and in the presence of 10 mM H2O2 during staining.

PAGE gel was neither inhibited by CN^- nor H_2O_2 and migirated as standard Mn SOD (lane e), therefore indicating the presence of a Mn SOD in the samples. The activity of the standard Fe SOD (lane f) was inhibited by the addition of H_2O_2 but not by addition of CN^- (Fig. 5B and 5C). No further change in the SOD enzyme activity pattern of maize pollen and anther extract, Baxtin or Polbax samples could be detected after H_2O_2 treatment (Fig. 5C) demonstrating that FeSOD is not present in the analyzed maize extracts. The NBT activity staining patterns are in agreement with the gelfiltration data i.e. the detected SOD enzyme activity in the analyzed maize preparations are located in the regions for low molecular weight protein molecules.

CONCLUSIONS

The presence of superoxide dismutase enzymes in extracts of pollen and anthers of *Zea mays* and in the related products Baxtin and Polbax was established after purification of the samples by a simple PVP precipitation. Superose gel filtration of the purified samples revealed that SOD enzyme activity was located around 30000 d. The enzyme activity was inhibited by CN^- indicating the presence of CuZnSOD. Polyacrylamide gel electrophoresis followed by NBT activity staining in the absence and presence of different inhibitors confirmed that the SOD enzyme activity in the pooled fractions from Superose gelfiltration of the freshly prepared maize extract, Baxtin and Polbax samples was due to CuZuSOD and MnSOD.

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OTHER SUPPORT:

GRAMINEX Flower Pollen Extract

Hay Fever and Pollen Tablets

By Einar Helander

Pollen preparations have been marketed since 1952. Until recently, they have not been used for medical purposes, but have been sold without restrictions as a commercial article. In a paper in this journal, Ask-Upmark (1960) has, however, suggested that pollen tablets should be used in the treatment of patients with prostatitis. It is still too early to state whether such therapy is rational, since a report is given of only a few patients. On the other hand, it can be expected that pollen preparations will be tested in the near future. The object of the present paper is therefore to clarify a problem of importance in this connection—one also raised by Ask-Upmark—i.e., can pollen preparations be administered to patients with pollen allergy without causing side-effects?

This question is important, since pollen allergy—usually in the form of hay fever—has been calculated to occur in 0.5-1% of all persons in Sweden. A number of different pollen types are responsible for these allergies. During a 10-year period (Sept. 1949 to Sept. 1959), 10.509 skin tests were made at the Allergy Department, Gothenburg, on patients who attended for various allergies. Routine tests were made for pollen grains of the following species: timothy, oxeye daisy, mugwort (*Artemisia vulgaris*), birch, alder, hazel and aspen. In a few cases additional tests were, for certain reasons, made with other pollen extracts. On the basis of these skin tests, provocation tests and data given by the patients, 2072 pollen allergies were diagnosed and subsequently treated.

The distribution of these allergies can be inferred from Tab. 1 (cf. Arnoldsson 1955). Since about one-third of the patients tested were allergic to more than one pollen species, the number treated is only just over half the total figure. The figures in this table can be regarded as representative of the Gothenburg region, whereas the distribution differs slightly in other parts of Sweden (Arner 1959).

The pollen preparations on the market (Cernelle, Cernident, Cernitin, Cernitol, Cerniton, Polloton, and Pollisan) contain both pollen husks and pollen extract. The husks are separated mechanically, and then heated with a view to decreasing the risk of allergy. The pollen extracts are obtained with water and organic solvents. In the different extraction procedures, up to 82% of the total nitrogen content of the pollen grains has been recovered. The various fractions are evaporated, and combined into a substance denoted as Cernitin.

According to statements from the manufacturers, the following pollen species are used in the preparation.

1. Timothy 26%
2. Maize 26%
3. Rye 19%
4. Hazel 6%
5. Sallow 6%
6. Aspen 6%
7. Oxeye daisy 6%
8. Pine 5%

It is already mentioned that allergies to timothy, oxeye daisy, hazel and aspen are common in the Gothenburg region. Allergy to sallow is not uncommon in other parts of Sweden in which it grows more

extensively (Arner 1959). Allergy to rye pollen is relatively rare, and that to pine pollen still more rare. Allergy to maize pollen is unknown in Sweden, but occurs in the U.S.A. (Urbach & Gottlieb 1946).

Present Investigation[1]

The composition of the pollen preparations gives good reason to investigate whether they can produce allergic symptoms. Several allergens cause allergic symptoms on oral administration, but the literature contains no data no whether this applies to pollen or preparations of it.

The tests were made on 25 patients who were allergic to pollen, but were healthy in other respects (see Tab. 2). The pollens to which these patients were allergic can be inferred from the table. The results of the tests were graded as follows. Histamine (1:10,000) was used as the positive control and 0.9% NaCl as the negative, the results being given in proportion to the area of the wheal produced by histamine, which is denoted as 3. Thus, 1 = a wheal with an area $^1/_3$ as large, 2 = $^2/_3$ of the area, 6 = twice as large, etc.

[1] The pollen preparations were kindly placed at my disposal by AB Cernelle, Vegeholm.

Tab. 1. Pollen allergies diagnosed at the Allergy Department, Gothenburg, 1949-1959.

Timothy and related grasses	913
Birch	358
Oxeye daisy and related plants	330
Alder	164
Hazel	150
Aspen	143
Rye	11
Fir	2
Reeds	1

1. Skin Tests with Extract of Pollen Tablets and Cernitin

After removing the sugar-coating, the Cernelle tablets were broken up and extracted with 5 parts of 0.9% NaCl, during vigorous shaking, for 2 hours on each of two consecutive days. This solution was sterile-filtered and then used for the tests. So-called cernitin, diluted to 1:10 with 0.9% NaCl, was used in the same way. Here as well, histamine was used as the positive control and 0.9% NaCl as the negative. The results were graded as described above. The extracts were tested on non-allergic subjects with negative results.

2. Demonstration of Antigen According to Praussnitz & Küstner

Venous blood was drawn from the 25 patients in question. After centrifugation, 0.1 ml of serum from each patient was injected intradermally into at least two healthy, non-allergic subjects. The latter had been given 5-25 Cernilton tablets on an empty stomach 60-90 minutes before the experiments. The results were graded as already started (Tab. 2).

3. Direct Administration of Pollen Tablets to Patients with Pollen Allergy

Each of the 25 patients with pollen allergy was given a test dose of one tablet of Cerniton. After one hour, a further four tablets were given, and somewhat later on the same day an additional 20 tablets on an empty stomach.

Tab. 2.

		Age	Skin Test for Pollen								Skin Test		Inverse Prauss.-Küst.			Reaction on oral adminis. No. of Tablets		
No.	Sex	Yrs	Ph	Be	Pr	Al	Co	Po	Ar	Se	Pt	Pe	5	15	25	1	5	25
1	F	19				2	3				2	2			(1)			
2	M	38		7							2	4		(1)	1			
3	M	21	3	6							2	3						
4	F	24		3	5						3	3		(1)	1			a
5	M	15		4	3						2	1			(1)			
6	F	45			4						2	1			(1)			
7	M	34	4		3						3	4						
8	M	53	5		3						1	3						b
9	M	23	4		3					4	5	7						
10	M	25	4								2	4			(1)			
11	M	35	5	5							3	5						
12	M	57	7	4							3	5						
13	F	18	5	5	6	4	3	4			3	4						
14	M	39	5								4	5						
15	F	39			3						1	1			(1)			
16	M	28			6			6			2	3						
17	M	23	5	3							3	5			1			
18	M	17	4	8							3	5			(1)			
19	F	26			4			5			2	3						
20	F	22	6								3	5						
21	M	17	4								2	3						
22	F	52			5				3		2	4						
23	M	14	7								4	5			(1)			
24	M	31	5	5		3	1				2	4			(1)			
25	F	48	6	4							4	5						

Ph = timothy; Be = birch; Pr = oxeye daisy; Al = alder; Co = hazel; Po = aspen; Ar = mugwort (*Artemisia vulgaris*); Se = rye; Pt = extract of pollen tablets; Pe = Cernitin. For grading of cutaneous reactions: see text.

a Inapp. incr. coryza.
b Flatulence.

Results

The results of the experiments are recorded in Tab 2.

The skin tests, both will extract of pollen tablets and cernitin, showed that the preparations contain extremely potent allergens. In most cases, a very large wheal appeared. It can be mentioned that a patient who was allergic to birch only (pollen preparations do not contain birch pollen) also had positive reactions. I have been unable to find any explanation of this cutaneous reaction.

When the pollen preparation was administered orally the so-called inverse Praussnitz-Küstner test showed that a small but sufficiently large quantity of antigen was absorbed in some cases. The reactions were, however, inappreciable in the large majority of cases, and a definite reaction occurred in three patients only. The reactions denoted as (1) may have been unspecific.

In the cases with a definite reaction occurred, the reaction was nevertheless slight. Thus, despite the large quantity of tablets ingested, only small amounts of pollen antigen were absorbed. For this reason, the preparation might possibly be used in attempting oral desensitization in hay fever.

The tests with administration of pollen tablets showed that the incidence of side-effects was low, even with large doses. One patient stated that the large number of tablets produced flatulence, and another that he seemed to feel some increased coryza, persisting for about 12 hours. The patient's complaints were regarded as so negligible that no therapy was indicated.

Unfortunately, the preparation could not be tested in patients with allergy to maize, pine, or sallow pollen. However, in Sweden these allergies play an insignificant or no role. Moreover, there is no reason to believe that they involve any essential differences.

To sum up, it can be stated that the experiments have shown the following:

1. The pollen tablets contain highly concentrated pollen antigens, which are not inactivated by the technical procedure used in their preparation.
2. On oral administration of pollen preparations, the antigen or components of it may be absorbed.
3. Absorption is, however, so slight that no risk of serious complications seems to exist, even if large doses are taken by subjects with pollen allergies. Consequently, hay fever is not a contraindication in those cases in which it is desired to test the effect of pollen preparations in, e.g., prostatitis.

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Opinion on the action of Cernitin

We have been requested to investigate the preparations designated as Cernitin T60 and Cernitin GBX with respect to their therapeutic effects.

Clinical observations indicate that these preparations are capable of effecting a cute of infections caused by bacteria or virus.

The mode of action of these preparations appears to be highly complex. Nevertheless, it seemed worthwhile to elucidate whether the subjective improvement could be verified by objectively measurable effects.

The effect on bacterial toxins, on enzymes and on antibodies can be satisfactorily demonstrated experimentally in vitro and in vivo.

I. **Effect of Cernitin T 60 and Cernitin GBX on streptolysin**

Method:

A streptolysin-0 solution in physiological saline solution containing 40 streptolysin-0 units per ml was prepared. Cernitin T 60 was added to this streptolysin solution to give final concentrations in the toxin solution of 0.1%, 0.5%, 1% and 10%.

Cernitin GBX was dissolved in distilled water containing 20% polyethylene glycol 400, giving a solution containing 1% Cernitin GBX. This 1% solution was treated with lyophilized streptolysin-0, so that a final concentration of 40 units per ml was obtained.

Owing to the sensitivity of streptolysin to heat, the mixture of toxin and Cernitin was stored in a cooler at +4° C. The reaction was permitted to proceed for various intervals of time, after which the activity of the streptolysin as determined. A streptolysin-0 solution which did not contain Cernitin was stored under identical conditions and served as a reference standard.

Loss of streptolysin activity was determined by treating the streptolysin test-sample and the streptolysin control solution with an equal part of sodium thioglycolate (which served as reducing agent) and three parts of a physiological saline solution which had been diluted by 1:5. After the mixing the streptolysin test-sample and the streptolysin control solution remained 10 minutes at room temperature and the toxin activity was measured as follows:

Tube No.	Reduced streptolysin	Physiological saline solution	Buffer solution	2% Blood cell suspension
1	0.5 ml		0.5 ml	0.25 ml
2	0.45 ml	0.05 ml	0.5 ml	0.25 ml
3	0.4 ml	0.1 ml	0.5 ml	0.25 ml
4	0.35 ml	0.15 ml	0.5 ml	0.25 ml
5	0.3 ml	0.2 ml	0.5 ml	0.25 ml
6	0.25 ml	0.25 ml	0.5 ml	0.25 ml
7	0.2 ml	0.3 ml	0.5 ml	0.25 ml
8	0.15 ml	0.35 ml	0.5 ml	0.25 ml
9	0.1 ml	0.4 ml	0.5 ml	0.25 ml
10	0.05 ml	0.45 ml	0.5 ml	0.25 ml

As seen from the Table, a series of test tubes was prepared into which portions of toxin solution were pipetted, the volume of each portion decreasing progressively by 0.05 ml and amounts of physiological saline solution then being added to each tube to make up a total volume of 0.5 ml. To this solution was added 0.5 ml of a buffer solution (composition: 1.45 g KH_2PO_4; 7.6 g Na_2HPO_4. $2H_2O$; 4.8 g NaCl; aqua dist. 1 lit.) and 0.25 ml of a 2% blood cell suspension.

Using this experimental equipment, it was possible to test, both qualitatively and quantitatively, the deleterious effect of the residual amounts of toxic substances by means of various concentrations of active material, the time of action of the detoxicating substances being taken into account. In addition, the preparation of a series of dilutions of toxin and Cernitin permitted detection of even partial detoxication of the streptolysin-0, this being of great value to the full exploitation of the detoxicating effect of Cernitin T 60 and Cernitin GBX.

The haemolysis was noted after immersion for 45 minutes in a water-bath at 36° C.

Result:

a. *Cernitin T 60*

Forty units of streptolysin-0 in 1 ml 10% or 1% solution of Cernitin T 60 were completely inactivated within 30 minutes at +4° C.

In a 0.5% Cernitin T 60 solution, the destruction of the blood cells by streptolysin was almost completely arrested after 2 hours at +4° C (see Table 1). On the other hand, a 0.1% Cernitin T 60 solution could not suppress the effect of the toxin even after 10 days at +4° C (see Table 2).

b. *Cernitin GBX*

Inactivation of streptolysin-0 commenced in a 1% Cernitin GBX solution only after two days of exposure at +4° C. On the ninth day, the streptolysin (40 units per ml) was completely inactivated (see Table 3.)

II. **Effect of Cernitin T 60 and Cernitin GBX on staphylolysin**

Method:

Staphylolysin was introduced into a 1% Cernitin T 60 solution and into a 1% Cernitin GBX solution in amounts which gave final concentrations of 1 unit per ml.

Both Cernitin T 60 and Cernitin GBX were diluted with a 1% glucose broth and 20% polyethylene glycol 400 was also added to the latter substance. Our choice of glucose broth for the staphylolysin experiments was dictated by the fact that the toxin remains active for a longer time in this medium than in physiological saline solution. Measurements of the toxic activity of this staphylolysin preparation at 37° C were made at various time intervals.

A staphylolysin solution of the same concentration, treated in an identical manner, served as a reference standard.

The toxic activity of the solutions was determined as follows:

A series of 10 reagent tubes was prepared, each containing 1 ml of a 1% glucose broth. One ml of the mixture of toxin and Cernitin (or of the toxin control solution) was introduced into the first tube.

After thorough mixing, 1 ml of this solution was transferred to the second tube. This procedure was then repeated throughout the entire series until a row of geometrically decreasing concentrations of the toxin and Cernitin preparation and of the control solution was obtained. One ml of solution from the last tube was discarded. One ml of a 2% rabbit erythrocyte suspension was pipetted into each tube. The tubes were then immersed in a water-bath at 37° C and the haemolysis was recorded after two hours.

Result:

No inactivation of the staphylolysin by 1% Cernitin T 60 or by 1% Cernitin GBX solution could be observed even after 10 days' exposure at 37° C.

III. **Determination of the influence of Cernitin T 60 and Cernitin GBX on enzymes**

A. *Urease:*

One g of Cernitin T 60 was added to 100 ml of a urease solution containing 1 mg urease per ml. (This procedure was repeated with Cernitin GBX). These solutions, as well as a control solution formulated in an identical manner but without addition of Cernitin, were stored at room temperature.

After 1, 2, 5 and 24 hours, the activities of the urease preparations were determined according to the method recommended by the firm of C.F. Boehringer and Sons, Mannheim, for their reagents. The test procedure is appended to the Tables.

The sera used had the following urea concentrations, expressed in mg %:

26	37	67	170	308	and
27	40	68	220	320.	
35	65	160	256		

Result:

Neither Cernitin T 60 nor Cernitin GBX were capable of affecting the activity of urease.

The results obtained in two experiments are presented in Table 4.

B. *Acid phosphatase:*

To test the influence of Cernitin on acid phosphatase, preparations of serum maintaining the following activities, expressed as mMoles of acid phosphatase, were prepared:

1.95	3.0	5.2	11.5	
2.4	4.5	6.0	24.0	and
2.6	5.1	7.1	36.0	

Cernitin T 60 or Cernitin GBX was added to the sera so that the final concentrations in the serum amounted to 1%. The preparations were then incubated at 37° C and the activites of the acid phosphatase were determined after 1, 2, 4, and 6 hours.

Serum prepared and treated in an identical manner, but without the addition of Cernitin, was used as a reference standard.

The acid phosphatase was essayed according to the method recommended by the firm of C.F. Boehringer and Sons, Mannheim, for their reagents. The test procedure is appended to the Tables.

Results:

Cernitin T 60 and Cernitin GBX were added to solutions of acid phosphatase of various activities to give final concentrations of 1% This concentration of Cernitin T 60 or Cernitin GBX was incapable of influencing the activity of the acid phosphatase even after 6 hours of exposure at 37° C.

C. *Glutamate pyruvate transaminase:*

To test the influence of Cernitin on glutamate pyruvate transaminase, preparations of serum maintaining the following activities of the enzyme, expressed as mU, were prepared:

14	40	93	185	
22	62	123	227	and
31	85	156	333.	

Cernitin T 60 and Cernitin GBX was added to the sera to give final concentrations of 1%. The serum-Cernitin-mixing was then incubated at 37° C and the activities of the enzymes were determined after 1, 2, 4 and 6 hours.

Serum prepared and treated in an identical manner but without the addition of Cernitin was used as a reference standard.

The glutamate pyruvate transaminase activity was determined according to the method recommended for their reagents by the firm of C.F. Boehringer and Sons, Mannheim. The test procedure is appended to the Tables.

Result:

Cernitin T 60 and Cernitin GBX were added to sera with different glutamate pyruvate transminase activity to give final concentrations of 1%. This concentration was not capable of affecting the glutamate pyruvate transminase activity even after 6 hours of exposure at 37° C.

Summary:

Clinical observations indicate that cases of inflammation caused by bacteria or virus could be favorably influenced by Cernitin.

Although the action of these preparations seems to be highly complex, attempts were made to achieve an objective appraisal of their effect on bacterial toxins and on enzymes occurring in the human organism.

It was found that the destruction of blood cells caused by treptococcal toxins could be arrested by both Cernitin T 60 and Cernitin GBX. Cernitin T 60 was especially active, a 1% solution leading to inactivation of a very high streptolysin concentration within half an hour. Even a 0.5% solution of Cernitin T 60 could inactivate large doses of the toxin after 4 hours at 4° C.

Neither a 1% solution of Cernitin T 60 nor a 1% solution of Cernitin GBX had any effect on the staphylococcal toxins which caused the destruction of blood cells.

Neither Cernitin T 60 nor Cernitin GBX appeared to have any effect on the enzymes: urease, acid phosphatase or glutamate pyruvate transaminase.

(Priv. Doz. Dr. med. M. Kienholz)

Head Physician.

Table 1.

Inactivation of streptolysin-0 in a 0.5 % solution of Cernitin T 60 at 4° C.

For dilution of the streptolysin-0 solution, see p. 3.

Time of effect	Tube numbers 1	2	3	4	5	6	7	8	9	10
Control soln. 24 hours	++++	++++	++++	++++	++++	++++	++++	++++	ø	ø
30 min.	++++	++++	++++	+	(+)	ø	ø	ø	ø	ø
1 hour	++++	++++	++++	++	++	ø	ø	ø	ø	ø
2 hours	+	++	ø	ø	ø	ø	ø	ø	ø	ø
4 hours	+	+	ø	ø	ø	ø	ø	ø	ø	ø
24 hours	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø

++++ = complete lysis of rabbit erythrocytes (fully active toxin).
+++ = 75 % of erythrocytes are haemolyzed (toxic activity reduced by 25 %).
++ = 50 % of erythrocytes are haemolyzed (toxic activity reduced by 50 %).
+ = 25 % of erythrocytes are haemolyzed (toxic activity reduced by 75 %).
ø = No haemolysis (toxin is inactive).

Table 2.

Inactivation of streptolysin-0 in a 0.1 % solution of Cernitin T 60 at 4° C.

For dilution of the streptolysin-0 solution, see p. 3.

Tube numbers.

Time of effect	1	2	3	4	5	6	7	8	9	10
Control soln. 10 days	++++	++++	++++	+−++	++++	++++	++++	+−	Ø	Ø
10 days	++++	++++	++++	+−++	++++	++++	+	Ø	Ø	Ø

++++ = Complete lysis of rabbit erythrocytes (fully active toxin).
+++ = 75 % of erythrocytes are haemolyzed (toxic activity reduced by 25 %).
++ = 50 % of erythrocytes are haemolyzed (toxic activity reduced by 50 %).
+ = 25 % of erythrocytes are haemolyzed (toxic activity reduced by 75 %).
Ø = No haemolysis (toxin is inactive).

Table 3.

Inactivation of streptolysin-0 in a 1 % solution of Cernitin GBX at 4° C.

For dilution of the streptolysin-0 solution, see p. 3.

Tube numbers.

Time of effect	1	2	3	4	5	6	7	8	9	10
Control soln. 9 days	++++	++++	++++	++++	++++	++++	++++	++++	Ø	Ø
1 days	++++	++++	++++	++++	++++	++++	++++	Ø	Ø	Ø
2 days	++++	++++	++++	++++	++++	++++	Ø	Ø	Ø	Ø
5 days	++++	++++	++++	+−++	++++	++++	Ø	Ø	Ø	Ø
7 days	++++	++++	++++	++++	++++	Ø	Ø	Ø	Ø	Ø
9 days	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø

++++ = Complete lysis of rabbit erythrocytes (fully active toxin).
+++ = 75 % of erythrocytes are haemolyzed (toxic activity reduced by 25 %).
++ = 50 % of erythrocytes are haemolyzed (toxic activity reduced by 50 %).
+ = 25 % of erythrocytes are haemolyzed (toxic activity reduced by 75 %).
Ø = No haemolysis (toxin is inactive).

Table 4.

Determination of urea with urease preparations containing 1 % of Cernitin T 60 or of Cernitin GBX.

The urea content is given in mg %.

Time of effect of Cernitin on urease at room temperature.	Urease + Cernitin T 60	Urease + Cernitin GBX	Urease without Cernitin (control)
1 hour	26	25	24
2 hours	25	26	25
5 hours	25	24	24
24 hours	23	24	24
1 hour	308	309	311
2 hours	310	311	309
5 hours	309	311	312
24 hours	292	295	293

Pharmacological and Toxicological Tests

University of Bologna

Institute of Pharmacology Pharmacodynamics and Toxicology

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Doctor Bruno Manica of Verona sent us a proprietary preparation, made up into sugar-coated tablets, named CERNILTON, in order that we should carry our pharmacological and toxicological tests.

The composition of the preparation is as follows:

<u>One tablet contains:</u>

Cernitin T60…….60 mg

Cernitin GBX_1…….....3 mg

Excipients…….q.s.

The clinical use proposed for the preparation is that of an anti-inflammatory agent, in a dose of 2-4 tablets/day.

The studies in our Institute were extended in two directions: first a group of experiments was carried out to estimate the activity of CERNILTON, by comparison with known drugs. Having established its activity we went on to study its toxicity in order to estimate the therapeutic coefficient.

EXPERIMENTS AND RESULTS

1. ACTIVITY a) carrageenin-induced oedema test in the normal and the adrenalectomized rat.
   b) cotton pellet granuloma test.

2. TOXICITY a) acute
   b) chronic
   c) foetal
   d) anaphylactic action

1. ACTIVITY

a) carrageenin-induced oedema in the normal and adrenalectomized rat.

In order to study carrageenin-induced oedema, the test preparation, administered by gastric intubation, was compared with phenylbutazone and indomethacin. The doses administered are reported in table 1, together with the results. Local oedema from carrageenin in the normal and adrenalectomized rat was produced, in the animal's paw, by means of the injection in the plantar region of 0.1 ml of a 0.5% solution of carrageenin in 0.9% NaCl.

The volume of the paw was measured in the individual rats with a gauge, three hours after the injection of the carrageenin.

The experiment was carried out on male rats of weight around 220 G, divided into three groups, two of which were used for the comparative assessment with indomethacin and phenylbutazone.

TABLE 1

Percentage inhibition of carrageenin-induced oedema in rat's paw.

Substance Tested	Dose	Number of Animals	Mode of Administration	% inhibition
Cernilton	$^1/_2$ tab/kg	10	Oral	33.7
	1 tab/kg	10	Oral	44.2
	1.5 tab/kg	10	Oral	56.8
	2 tab/kg	10	Oral	65.4
Indomethacin	0.5 mg/kg	10	i.p.	20.4
	1.0 mg/kg	10	i.p.	34.5
	2.0 mg/kg	10	i.p.	38.3
	3.0 mg/kg	10	i.p.	46.7
Phenylbutazone	25 mg/kg	10	i.p.	26.2
	50 mg/kg	10	i.p.	39.3
	100 mg/kg	10	i.p.	52.6
	200 mg/kg	10	i.p.	60.7

Examination of the data reported in table 1 shows that CERNILTON demonstrates inhibitory activity on carrageenin-induced oedema.

The test of the inhibition of carrageenin-induced oedema was repeated on the adrenalectomized rat, in order to avoid the liberation of corticosteroids. A single dose of 1.5 tablets/kg of CERNILTON was administered to a group of 10 rats by gastric intubation. From this experiment it was apparent that the oral administration of 1.5 tablets/kg of CERNILTON produces an inhibition of oedema amounting to 53.2%. This results is of the same order of magnitude as that found in the non-adrenalectomized rat (56.8%).

This finding enables stress to be excluded as the cause of anti-inflammatory activity by means of release of corticosteroids.

b) Cotton pellet granuloma test.

This test, consisting in the subcutaneous implantation of cotton pellets of initial weight 15 ± 0.2 mg, sterilized at 120° C for 2 hours and moistened with penicillin (50 µg/pellet), was carried out under light ether anesthesia in male rats. After 8 days the pellets were removed, dried for 24 hours at 60° C and then weighed again. The experiment was performed on 40 rats weighing around 200 G, divided into 4 groups, respectively: controls, treated with CERNILTON, treated with Indomethacin, and treated with Phenylbutazone.

In table 2 below the data are expressed in terms of percentage inhibition of the weight of the granuloma compared with the untreated controls.

The anti-inflammatory action of CERNILTON is apparent, in this test, at a dose of 1 tablet/kg.

TABLE 2

Percentage inhibition of the “cotton pellet” granuloma.

Substance Tested	Dose	Number of Animals	% inhibition
Cernilton	1 tab/kg	10	36
Indomethacin	1.6 mg/kg	10	45.2
Phenylbutazone	100 mg/kg	10	42.7

2. TOXICITY

a) Acute Toxicity

The LD_{50} was estimated in the rat and the mouse after oral administration of the test preparation. The experiment was carried out using the logarithmic method of C.S. Weil (Biometrics, Sect. 1952) based on the administration of doses increasing in geometrical progression.

The experiment was carried out in 4 groups of 10 animals each, who were observed for a period of 48 hours after drug administration. The results obtained are reported in the following tables.

TABLE 3

Acute toxicity of CERNILTON in male Sprague-Dawley rats of weight 200-220 G.

Doses: tablets/kg	Mode of Administration	Number of Animals per group	Mortality	LD_{50}
1	Oral	10	0/10	> 8 tab/kg
2	Oral	10	0/10	
4	Oral	10	0/10	
8	Oral	10	0/10	

TABLE 4

Acute toxicity of CERNILTON in the male albino mouse weighing 22-25 G.

Doses: tablets/kg	Mode of Administration	Number of Animals per group	Mortality	LD_{50}
1	Oral	10	0/10	> 8 tab/kg
2	Oral	10	0/10	
4	Oral	10	0/10	
8	Oral	10	0/10	

b) *Chronic toxicity*

The test was performed on the Sprague-Dawley rat and the New Zea and rabbit. Treatment by the oral route was continued for a period of 180 consecutive days, the test preparation being mixed with the food ground up and made into a paste with water. Suitable containers prevented the dispersion of the food and ensured the total consumption of the substance given to the animals. The condition of the animals was determined periodically, and checks carried out on their weight gain, blood picture and renal function. At the end of the treatment the above observations were completed with determinations of the white cell formula and liver function, and macroscopical and microscopical examination of the principal organs.

A. Test on the RAT

20 Sprague-Dawley rats were used, divided into 2 groups of 10 each in the following way:

Group 1: controls
Group 2: treated with CERNILTON in a dose of 1 tablet/kg. The results obtained are as follows:

TABLE 5

Increase in body weight in the control rats and those treated with CERNILTON. Mean values ± S.E.

Days of Treatment	Controls	Treated
0	102 ± 1.4	101 ± 1.8
30	162 ± 2.7	160 ± 2.6
60	261 ± 3.8	262 ± 4.1
90	320 ± 4.9	315 ± 5.3
120	360 ± 5.7	365 ± 5.8
180	395 ± 6.1	393 ± 6.7

Examination of table 5 shows no significant variation in body weight of the animals treated with CERNILTON with the control animals.

BLOOD PICTURE

Erythrocyte and leukocyte counts were carried out periodically during the treatment of the rats with the test preparation and at the end of this treatment. The count, performed by means of a Thoma-Zeiss chamber on samples of blood taken from the animals' tails, gave the following results.

Adrenal: normal cortex and medulla.
Stomach and intestine: no lesions seen in the mucosa, submucosa or muscle layers.

CONCLUSIONS:

It is clear that the treatment with the preparation CERNILTON, continued over a period of 180 days, has no inhibitory effect on the body growth of the rat, nor on the blood picture, nor on hepatic or renal function. There are no resulting signs of damage to the principal organs. For this reason it is concluded that the preparation CERNILTON is not toxic to the Sprague-Dawley rat, even when treatment is for prolonged periods.

B. Test on the rabbit

The test was carried out on 20 New Zealand rabbits divided into two experimental groups of 10 animals each in the following way:

Group 1: Control
Group 2: Treated with CERNILTON in a dose of 1 tablet/kg.

The test preparation was mixed with the diet, ground up and made into a paste with water. The control animals received only their food ground up and made into a paste.

The treatment was started in young animals, 50 days old, and continued for 180 days.

The results obtained are set out in the following tables.

TABLE 10

Body growth of the rabbits in the two experimental groups. Mean values per group ± S.E. expressed in Kg.

Days of Treatment	Controls	Treated
0	1.12 ± 0.011	1.18 ± 0.015
30	1.40 ± 0.024	1.43 ± 0.018
60	1.96 ± 0.032	1.95 ± 0.029
90	2.15 ± 0.047	2.18 ± 0.051
120	2.68 ± 0.059	2.73 ± 0.071
180	3.10 ± 0.064	3.17 ± 0.063

The body growth of the rabbits treated with CERNILTON does not differ significantly from that of the control animals.

TABLE 11

Blood picture. Erythrocytes (10^4 per mm^3) and leucocytes (10 per mm^3) in the blood of the rabbits. Mean values ± S.E.

Days of Treatment	Erythrocytes	
	Controls	Treated
0	974 ± 12.8	972 ± 14.1
60	979 ± 13.2	939 ± 17.1
120	942 ± 13.9	1012 ± 14.1
180	1004 ± 15.1	998 ± 15.9

Days of Treatment	Erythrocytes	
	Controls	Treated
0	648 ± 7.1	628 ± 6.7
60	610 ± 5.2	607 ± 4.3
120	624 ± 6.7	618 ± 7.2
180	643 ± 7.5	639 ± 7.8

The counts were carried out with a Thoma-Zeiss cell-counting chamber.

The values obtained in the animals treated with the test preparation do not differ from the normal.

The search for albumin and occult blood in the urines of the treated animals, carried out periodically during the treatment, gave consistently negative results.

Examination of peripheral blood films, prepared at the end of the treatment with the test preparation, enabled the mean values of the white cell formula to be worked out for each group, as shown in the following table.

TABLE 12

White cell formula of the rabbits in the two experimental groups. Mean values per group ± S.E.

	Lymphocytes	Monocytes	Granulocytes		
Group	%	%	Neutrophils	Eosinophils	Basophils
Controls	48.7 ± 1.2	4.9 ± 0.27	43.5 ± 1.62	1.9 ± 0.05	1.0 ± 0.01
Treated	47.8 ± 1.7	4.7 ± 0.30	44.5 ± 1.72	1.8 ± 0.06	1.2 ± 0.02

The determination of the serum GOT and GPT activities of the animals in the experimental groups was performed at the end of the period of chronic treatment by taking samples of blood from the marginal vein of the ear of each animal.

The test was performed with the colorimetric test of the Boehringer Company of Milan. The results obtained are set out in the following table.

TABLE 13

Serum GOT and GPT activities of the rabbits in the two experimental groups at the end of the chronic treatment. Mean values ± S.E.

Enzymatic Activity	Controls	Treated
SGOT: mU/ml	32.1 ± 2.27	31.5 ± 2.18
SGPT: mU/ml	17.8 ± 1.30	16.9 ± 1.32

Analysis of the above results shows no significant differences in the values obtained in the treated animals compared with those of the controls.

Macroscopical examination of the rabbits, sacrificed by carotid section at the end of the treatment, did not demonstrate any signs of damage to the organs of the animals subjected to chronic treatment with the preparation CERNILTON. Entirely normal appearances were found in the liver, kidney, spleen, heart, lungs, stomach, intestine and genital system.

The fresh weight of the principal organs in reported in the following table.

TABLE 14

Fresh weight of the organs of the rabbits in the two experimental groups. Mean values ± S.E.

Group	Liver G	Spleen G	Kidney G	Heart G	Adrenal mg
Controls	154 ± 1.65	1.18 ± 0.10	14.5 ± 0.19	8.8 ± 0.21	195 ± 3.9
Treated	148 ± 1.07	1.22 ± 0.09	13.8 ± 0.17	9.5 ± 0.18	186 ± 4.6

Histological examination was carried out on preparations of the principal organs fixed in formalin and stained with hematoxylin-eosin. No signs were found of toxicity or poor tolerance of the preparation given.

The relevant findings in the treated animals can be summarized as follows:

Liver: normal structure of the hepatic lobules. Normal liver cells with rounded nucleus in the centre of the cytoplasm. Normal vascular network and biliary ducts.
Kidney: renal pelvis clear. No cloudy swelling seen in the renal glomeruli or in the proximal or distal tubules. No interstitial infiltration with small cells seen.
Heart: nothing remarkable.
Adrenal: normal cortex and medulla.
Stomach and intestine: no lesions seen.

CONCLUSIONS:

From the results reported above it can be seen that continued treatment for 180 days with the preparation CERNILTON has no inhibitory effect on the growth and development of the rabbit. The blood pictures is not affected, nor are renal of hepatic function. No manifestations of toxicity are seen in the principal organs.

It is concluded that the preparation CERNILTON is not toxic to the New Zealand rabbit when given orally even for as long as 180 days.

c) Foetal toxicity

The evaluation of foetal toxicity and the search for possible teratogenic properties of CERNILTON were carried out by means of two experiments: the first on the Sprague-Dawley rat, and the second on the New Zealand rabbit. Treatment was given by mouth during the organogenetic period of pregnancy, the test preparation being administered by gastric intubation in three different doses.

Examination of the foetuses was carried out at birth. Account was taken of their number, their vitality and their body weight. The skeleton was examined after rendering the soft tissues transparent and staining the bony tissue with alizarin red. After the end of pregnancy all the animals were sacrificed in order to look, by direct examination of the walls of the uterine cornua, for possible signs of resorption.

A. Test on the rat

The test was carried out on 40 female and 20 male adult Sprague-Dawley rats. The animals were distributed in 20 cages each containing 2 females and one male. The mating period lasted for 5 days during which the males were rotated daily between the cages in such a way that during the mating period two females were in the company of different males. By this procedure a percentage of pregnancies of 50 to 70% were obtained.

The treatment with the test preparation was started immediately after the end of the mating period when the females were divided into 4 groups of 10 each. The four groups were constituted as follows:

Group 1: Controls
Group 2: Treated with CERNILTON in a dose of 1/2 tablet/kg by gastric intubation.
Group 3: Treated with CERNILTON in a dose of 1 tablet/kg by gastric intubation.
Group 4: Treated with CERNILTON in a dose of 1.5 tablets/kg by gastric intubation.

The treatment was carried out daily from the first to the 15th day of pregnancy.

TABLE 15

Foetal toxicity of Cernilton in the rat.

Group	1		2		3		4	
Number of Animals per group	10 F	5 M	10 F	5 M	10 F	5 M	10 F	5M
Number of Pregnancies	6		7		5		7	

Findings:

Total number of foetuses	67	84	56	77
Mean number of fetuses per litter	11	12	10	12
Mean foetal weight in G	5.97	6.07	6.15	6.09
Number of live births	67	82	56	76
Number of stillbirths	0	2	0	1
Resorptions	2	0	1	2
Number malformed	0	0	0	0

The results set out in table 15 were subjected to statistical analysis by carrying out a comparison between the various respective incidences in the 4 experimental groups according to the X^2 test. The following table shows the comparisons made.

TABLE 16

Statistical comparison of the results obtained in the 4 experimental groups.

	Parameters Compared	Calculated X^2	Significance
-	Pregnancy animals versus non-pregnant animals	1.7333	Not sig.
-	Foetuses per pregnancy	0.0285	Not sig
-	Live births versus stillbirths	2.7665	Not sig.
-	Resorptions	2.3315	Not sig.

Critical values for 3 degrees of freedom:

P 0.05 = 7.815 P 0.01 = 11.345

No significant differences were seen in the number of pregnancies, total number of fetuses, number of live births and still births, and number of resorptions in the 4 experimental groups. The preparation CERNILTON therefore shows no foetal toxicity or teratogenic activity in the Sprague-Dawley rat.

B*. Test on the rabbit*

The test was conducted on 20 adult female New Zealand rabbits that after mating were divided into 4 experimental groups of 5 each. The 4 groups were subjected to the following treatments:

Group 1: Control
Group 2: Treated with CERNILTON in a dose of 1/2 tablet/kg
Group 3: Treated with CERNILTON in a dose of 1 tablet/kg
Group 4: Treated with CERNILTON in a dose of 1.5 tablet/kg.

The treatment was carried out daily be gastric intubation and continued from the 1st to the 20th day of pregnancy. The results obtained are reported in the following table:

TABLE 17

Foetal toxicity of CERNILTON in the rabbit.

Group	1	2	3	4
Animals per Group	5	5	5	5
Number of Pregnancies	5	4	4	5
Findings:				
Total number of foetuses	43	34	31	37
Mean number of fetuses per litter	8	9	8	7
Mean foetal weight	51.5	49.7	51.8	50.2
Number of live births	43	33	31	36
Number of stillbirths	0	1	0	1
Number of resorptions	1	0	0	2
Number of malformations	0	0	0	0

In this experiment also the results obtained were subjected to statistical analysis by making a comparison between the various respective frequencies in the 4 experimental groups according to the X^2 test. The following table reports the comparisons made.

TABLE 18

Statistical comparison of the results obtained in the 4 experimental groups.

Parameters Compared	Calculated X^2	Significance
Pregnant animals versus non-pregnant animals	2.2222	Not sig.
Foetuses per pregnancy	0.0665	Not sig.
Live births versus stillbirths	2.1210	Not sig.
Resorptions	3.2468	Not sig.

Critical values for 3 degrees of freedom:

P 0.05 = 7.815 P 0.01 = 11.345

No significant differences were observed in the number of pregnancies, the total number of fetuses, the numbers of live births and stillbirths and the number of resorptions in the 4 experimental groups. It is therefore concluded that the preparation CERNILTON exerts no foetal toxicity or teratogenic activity on the New Zealand rabbit.

d) Anaphylactic action

The anaphylactic action was studied by means of the technique described by Pasteur – Valery Radot on page 83 of the treatise “Maladies allergiques”. This technique consists of the intravenous injection of a suspension of the test preparation, finely dispersed, 48 hours after sensitization with the same suspension given subcutaneously. A positive response is indicated by hypotensive collapse and haemorrhagic extravasations in the internal organs of the guinea-pig. The study was carried out on 30 adult guinea-pigs weighing around 500 G. Three subcutaneous injections of suspension of CERNILTON were given on 3 consecutive days. Subsequent injection intravenously of the suspension diluted 1/100 did not give rise, in the pre-treated guinea-pig, to manifestations of collapse. The internal organs of the animals, on examination at autopsy, did not show manifestations of haemorrhage.

In consequence of this sensitization phenomena on the part of the test preparation can be excluded, inasmuch as no greater incidence of anaphylactic phenomena is to be expected than may be found with any category of drug.

CONCLUSIONS

Given the consistently negative results of the tests of acute, chronic and foetal toxicity, the absence of anaphylactic action and the consistent inhibitory activity on the inflammation produced in two tests (carrageenin-induced oedema and cotton pellet granuloma) it can be concluded that the preparation CERNILTON can provide excellent results in its proposed clinical use as an anti-inflammatory agent.

Bologna 16 Apr, 1971.

OTHER SUPPORT:

GRAMINEX Flower Pollen Extract

Pollen as a Cholesterol-Lowering Agent

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Cernilton

Each capsule contains 60 mg of a water-soluble extract (Cernitin T 60) from flower-pollen and 3 mg of a lipid-soluble extract (Cernitin GBX). Flower-pollen has been purified of pollen-capsules and partially hydrolyzed, to obtain a pure preparation which does not give rise to allergic reactions. This preparation is different from bees-pollen that is more heterogeneous and more allergenic.

Cernitromb:

Each capsule contains Cernitin T 60, 120 mg and Cernitin GBX, 6 mg.

Both Cernilton and Cernitromb are registered as “naturmedel” (naturopathic remedies) by the Swedish State Medical Board.

Dosage: Four capsules daily for two months.

Analyses: Cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides were assayed in fasting plasma by routine methods at the Department of Clinical Chemistry, University Hospital, Lund. For fractionation of cholesterol a simple precipitation method was used.

Results

As evident from Table I a significant decrease of 11-13 percent was noted for plasma-cholesterol both with Cernilton and Cernitromb, perhaps slightly more so with Cernitromb. No change was seen in the placebo group.

The decrease was explained exclusively by a 17-18 percent decrease of LDL-cholesterol with no change or a slight increase for HDL-cholesterol. There was no significant change of plasma triglyceride levels. The preparations were well tolerated and no side-effects could be recorded.

TABLE I

Plasma lipid levels during treatment with pollen.
All values given as mmol/l.

		0 months	2 months	p-value
P-cholesterol	Placebo (n = 18)	7.73	7.77	n s
	Cernilton (n = 20)	7.67	6.81	< 0.001***
	Cernitromb (n = 20)	7.68	6.68	<0.001***
P-HDL-cholesterol	Placebo	1.29	1.32	n s
	Cernilton	1.10	1.21	< 0.05*
	Cernitromb	1.25	1.27	n s
P-LDL-cholesterol	Placebo	5.85	5.87	n s
	Cernilton	5.95	4.95	< 0.001***
	Cernitromb	5.74	4.73	< 0.001***
P-Tri-glycerides	Placebo	1.39	1.30	n s
	Cernilton	1.39	1.37	n s
	Cernitromb	1.94	1.84	n s

P-values denote comparison between time 2 months and 0 months.

n s = not significant

* = significant change at the 5 percent level.

*** = significant change at the 0.1 percent level.

Discussion

Our result supports those of Kosmider et al (6) as regards P-cholesterol levels. The Polish group found a more marked decrease with a higher dose (six capsules). Possibly, our results might indicate a slightly better effect with the higher dose.

The decrease in cholesterol noted in this study was explained by a decrease only in the LDL-fraction, sometimes called “the bad cholesterol”. This should imply a reduced risk for atheromatosis and cardiovascular disease. The fact that the HDL-fraction did not decrease or even increased slightly would tend to reinforce the positive effect of this treatment.

Pollen in the preparation used here has been used for many years. It has been shown to be effective in prostatitis and prostatic hyperplasia (7), to reinforce immunological defense mechanisms (8) and improve personal capacity (9). Flower-pollen, in conclusion, would be a very interesting natural product. Its effects on plasma cholesterol may come to be of great value, considering the very large need for a lowering of cholesterol levels in the population by means of simple and safe agents in addition to improved diet.

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CERNILTON

Initials	Sex	Age	Kolesterol 0-2		HDL-Kol. 0-2		LDL-Kol. 0-2		Triglycerides 0-2 months	
E.A.	F	70	7.50	6.08	1.20	1.39	6.00	4.25	1.40	0.98
S.A.	M	61	7.26	7.36	0.88	0.98	5.94	5.79	0.98	1.31
H.A.	F	70	7.54	7.24	1.32	1.66	5.10	4.96	1.55	1.37
S.E.B.	M	51	6.86	6.35	1.16	1.39	5.26	4.59	0.97	0.82
E.M.B.	F	55	7.30	7.19	1.40	1.17	5.86	5.55	0.70	1.04
B.B.	M	43	7.82	7.08	1.18	1.27	6.28	5.28	0.95	1.18
J.E.	F	70	7.92	6.49	1.39	1.58	5.73	4.49	0.92	1.01
B.G.	F	58	7.70	7.35	1.09	1.81	5.71	5.15	0.67	0.86
D.H.	F	70	8.67	7.55	0.97	1.14	6.74	5.34	2.14	2.37
A.J.	F	69	10.5	8.93	0.71	0.73	8.04	6.89	3.88	2.92
K.J.	M	60	7.48	5.77	1.27	1.21	5.76	4.25	1.00	0.69
S.J.	M	57	8.26	6.62	1.61	1.33	6.22	4.68	0.95	0.94
I.K.	M	58	7.54	5.83	0.91	1.07	6.06	3.92	1.27	1.86
G.K.	F	66	7.25	6.58	0.91	1.07	5.98	4.67	1.97	1.73
L.L.	M	53	6.50	6.16	0.91	1.13	4.76	6.60	0.95	0.96
L.N.	F	70	7.91	7.08	1.19	1.37	6.19	4.76	2.14	1.19
N.N.	M	70	7.85	7.36	1.00	0.99	6.13	5.69	1.28	1.37
E.P.	M	63	7.41	5.78	1.09	0.86	5.98	4.55	0.75	0.83
B.S.	M	63	6.60	6.10	1.10	1.16	5.20	4.66	0.80	0.62
K.S.	M	46	8.01	7.30	0.79	0.86	6.13	--	2.42	--
Mean			7.67	6.81	1.10	1.21	5.95	4.95	1.39	1.37
p			< 0.001		< 0.05		< 0.001		n.s.	

CENTRITROMB

Initials	Sex	Age	Kolesterol 0-2		HDL-Kol. 0-2		LDL-Kol. 0-2		Triglycerides 0-2 months	
I.A.	F	65	7.10	6.41	1.10	0.90	5.30	4.86	1.50	1.46
K.A.	F	62	8.05	6.72	1.10	1.10	6.94	4.88	2.96	1.99
U.A.	F	60	7.55	6.46	1.50	1.49	5.36	4.48	1.47	1.40
A.A.	M	69	7.52	6.21	1.44	0.95	5.49	4.51	1.31	1.66
B.B.	M	57	8.05	7.52	1.63	1.53	6.05	5.59	0.83	0.89
E.B.	M	51	6.60	6.83	1.40	1.44	4.80	5.00	0.96	0.86
I.B.	F	70	7.22	6.84	1.89	1.89	4.37	4.54	1.55	1.23
P.C.	M	65	7.81	6.53	0.72	0.93	5.99	4.50	2.33	2.74
B.H.	M	53	8.10	6.72	1.13	1.09	6.24	4.90	2.34	2.11
C.N.	M	45	6.50	6.15	0.67	0.58	2.92	--	5.36	6.04
H.N.	M	64	7.05	6.10	0.75	0.78	5.44	4.52	2.21	2.09
S.O.	M	65	6.50	5.30	0.91	0.90	3.83	3.18	3.04	2.72
K.O.P.	M	64	8.64	6.87	1.09	0.86	5.30	4.35	5.00	3.68
K.P.	M	60	7.44	6.50	1.58	1.40	5.45	4.61	0.90	1.09
N.P.	F	66	8.86	7.30	1.43	3.30	6.95	3.71	1.07	0.64
I.S.	F	58	8.01	7.37	1.69	1.63	5.84	5.30	1.38	1.30
G.S.	F	70	9.19	7.64	1.42	1.24	7.01	5.68	1.69	1.59
A.G.S.	F	62	7.30	6.04	1.40	1.31	5.35	4.25	1.23	1.07
Y.S.	M	60	7.70	6.80	1.20	1.20	6.20	5.13	0.80	1.04
M.S.	F	68	8.80	7.28	0.96	0.84	7.14	5.90	1.56	1.20
Mean			7.68	6.68	1.25	1.27	5.74	4.73	1.94	1.84
P			< 0.001		n.s.		< 0.001		n.s.	

PLACEBO

Initials	Sex	Age	Kolesterol 0-2		HDL-Kol. 0-2		LDL-Kol. 0-2		Triglycerides 0-2 months	
S.E.A	M	46	6.50	6.21	0.70	0.81	3.70	4.37	4.10	2.28
I.B.	F	68	7.38	8.05	0.94	1.07	5.58	5.72	1.92	2.81
K.E.B.	M	51	7.40	6.50	1.00	0.94	5.30	4.80	2.50	1.68
G.B.	F	57	7.32	7.97	1.75	1.86	5.25	5.71	0.71	0.89
M.D.	F	62	8.24	8.05	1.65	1.42	6.19	6.26	0.88	0.82
L.E.	M	56	7.51	7.91	1.03	1.20	5.81	6.15	1.48	1.24
L.G.	M	63	7.48	7.78	1.10	1.12	6.01	6.04	0.81	1.38
M.G.	F	52	7.26	7.54	0.99	1.32	5.72	5.67	1.23	1.23
A.I.	F	54	7.90	7.98	1.03	1.11	6.10	5.67	1.70	2.66
G.J.	M	59	8.48	8.58	1.21	1.08	6.44	6.86	1.84	1.41
C.J.	M	61	7.71	7.41	1.49	1.66	6.11	5.59	1.12	0.74
L.L.	F	70	7.11	6.86	1.05	1.13	5.81	5.27	1.40	1.02
B.L.	F	45	6.81	6.86	0.98	1.30	5.41	5.26	0.94	0.66
M.L.	F	63	9.01	8.91	1.87	1.59	6.83	7.02	0.69	0.67
I.N.	F	65	9.91	10.4	1.58	1.41	7.85	8.55	1.05	0.98
I.N.	F	68	8.10	8.04	1.51	1.57	6.01	6.01	1.30	1.02
K.S.	F	52	8.55	8.19	1.95	1.99	6.26	5.70	0.75	1.10
M.S.	F	68	6.60	6.64	1.31	1.20	4.98	5.08	0.68	0.80
Mean			7.73	7.77	1.29	1.32	5.85	5.87	1.39	1.30
p			n.s.		n.s.		n.s.		n.s.	

OTHER SUPPORT:

GRAMINEX Flower Pollen Extract

Salmonella-Escherichia coli / Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay (ABSTRACT)

Test Article	G-63 Powder
Author	Michael S. Mechhi, MS
Sponsor	Graminex 95 Midland Road Saginaw, Michigan 48603
Test Facility	Covance Laboratories Inc. 9200 Leesburg Pike Vienna, Virginia 22182-1699
Covance Study Number	7740-102
Genetic Toxicology Assay Number	27995-0-409OECD
Report Issued	28 June 2006
Page Number	1 of 50

Abstract

The objective of this study is to evaluate the test article G-63 Powder, and/or its metabolites for their ability to induce reverse mutations at the histidine locus in several strains of *Salmonella typhimurium*, and at the typtophan locus of *Escherichia coli* strain WP2*uvr*A, in the presence or absence of an exogenous mammalian activation system (S9) containing microsomal enzymes.

The doses tested in the mutagenicity assay were selected based on the results of a dose rangefinding study using tester strains TA100 and WP2*uvr*A (pKM101) and ten doses of test article ranging from 6.67 to 5000 µg per plate, one plate per dose, both in the presence and absence of S9 mix (see Protocol Deviations).

The tester strains used in the mutagenicity assay were *Salmonella typhimurium* tester strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* tester strain WP2*uvr*A. The assay was conducted with six doses of test article in both the presence and absence of S9 mix along with concurrent vehicle and positive controls using three plates per dose. The doses tested were 33.3, 100, 333, 1000, 3330, and 5000 µg per plate in both the presence and absence of S9 mix. The results of the initial mutagenicity assay were confirmed in an independent experiment.

The results of the *Salmonella-Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a Confirmatory Assay indicate that under the conditions of this study, the test article, G-63 Powder, did not

cause a positive increase in the mean number of revertants per plate with any of the tester strains either in the presence of absence of microsomal enzymes prepared from Aroclor^{IM}-induced rat liver (S9).

OTHER SUPPORT:

GRAMINEX Flower Pollen Extract

Streptolysin Inhibitory Factor in Pollen

ENDRE KVANTA

Institute of Chemistry, Teknikum, Växjö, Sweden

The starting material used for the isolation of a factor which inhibits the hemolytic activity of streptolysin-O is a water-acetone fraction (manufacturer's name: Cernitin spissum) of a standardized pollen mixture obtained from six plant species: *Zea maize, Pinus montana, Secale cereal, Phleum pratense, Alnus glutinosa,* and *Dactilus glomerata.* The mixture is produced for commercial purposes by AB Cernelle, Ängelholm, Sweden. The inhibition of streptolysin-O by treatment with the Streptolysin Inhibitory Factor (SIF) *in vitro,* and the chemical and physio-chemical properties of the inhibiting factor have been studied.

Streptolysin-O is a heat-stable, oxygen-sensitive bacterial toxin (a metabolic product of Streptococci), belonging to a group of enzymes known as hemolysins. Under physiological conditions, streptolysin-O hemolyzes erythrocytes.

The pharmacological effect of streptolysin in Streptococcus infections has aroused considerable medical interest in the toxin.[1] Very few reports have been published concerning the inhibition of the enzymatic effect of streptolysin. No specific inhibitor of the effect of streptolysin on red blood cells is known. Inhibitory effects of fat-soluble extracts from certain plant species have been described by Kienholtz,[2] who found that chamomile oil from whild chamomile (*Matricaria chamomilla)* and the oil of black radish (*Cochlearia armoracia)* inhibit streptolysin activity *in* vitro. Kienholtz[3] reported further, that lavender oil (*Lavendula spica*) inhibits streptolysin after a 3-day incubation period at 37°C, and that lavender oil is used medicinally in the treatment of certain bronchial disturbances.

The inactivation of streptolysin by treating with anti-streptolysin-O and trypsin has been described by Nakamura.[4] Belgova[5] has reported on studies which showed that EDTA did not have any enzyme-inhibiting effect on streptolysin, whereas cobalt nitrate at a concentration of no more than 10 ppm caused strongly reduced activity. Finally, Costa *et al.*[6] reported on studies which showed that streptolysin-O is inactivated by certain lysozymes, protamine sulphate, papain, and thymohistone.

STREPTOLYSIN INHIBITORY FACTOR

EXPERIMENTAL

1. Apparatus and equipment

The following apparatus was used in connection with gel filtration. The optical density of the eluate was measured continuously with an LKB 4701 A Uvicord I at 254.7 nm and recorded with an LKB 6520 Recorder. The fractions were collected with an LKB 7000 Ultro-Rac Fraction Collector. Spectrophotometrical measurements were carried out with a Beckman DB.

2. Purification of SIF

Fractionation with methanol. 5 ml Cernitin spissum was added slowly and under stirring to

100 ml methanol. The mixture was filtered after approx. 30 minutes shaking at room temperature. The supernatant was evaporated to approx. 3 ml by fan drying while stirring, and diluted to 5 ml with distilled water. (Cernitin spissum supernatant.) The supernatant and the sediment were tested for SIF activity.

Gel filtration on Sephadex. SIF in Cernitin spissum supernatant was purified by repeated gel filtration on a Sephadex G-15 column measuring 25 X 400 mm. The eluents used were 0.9% or 0.1% saline. After the first gel filtration run, the fractions belonging to the same UV peak were polled and tested for SIF activity. The SIF-active fractions were concentrated to the original sample volume and gel filtrated once again. The SIF-active fractions from the second gel filtration were concentrated and re-gel filtrated under the same conditions as above. The SIF-active fractions from the third gel filtration were concentrated and analyzed.

The active fractions (the pool) from the gel filtrations were analyzed with respect to the Folin intensity (Folin-Ciocalteau reaction), and the total carbohydrate content according to Dische.[7] The optical density of the solutions was read at 257 nm in a UV spectrophotometer.

3. Preliminary investigation of the physico-chemical properties of SIF

Heat stability. Cernitin spissum solutions, conc. 1:200, are incubated at 65°C and 100°C for 10 min. After cooling, the SIF activity of the solutions was investigated and compared with the activity of untreated SIF solution.

pH stability. The pH level of Cernitin spissum solutions, conc. 1:200, was adjusted with HCl to 2.0 or with sodium hydroxide solution to 11.5. The solutions were incubated at 37°C for 20 min, after which the pH values were adjusted to 5 (corresponding to the pH for untreated samples of Cernitin spissum). The SIF activity of the solutions was investigated and compared to the activity of untreated, incubated SIF solution.

Dialysis ability. Cernitin spissum solution, conc. 1:100, was dialyzed with the aid of a dialysis tube with a diameter of 20 mm (Union Carbide Corp.) against equal quantities of physiological saline at +4°C for 24 h. The SIF activities of the outer and inner solutions were investigated and compared with the activity of Cernitin spissum solution, conc. 1:200, stored at +4°C for 24 h, and with Cernitin spissum solution, conc. 1:200, stored in a dialysis tube at +4°C for 24 h.

Molecular weight determination. The preliminary value for the molecular weight of SIF was calculated according to Carnegie[8] and Andrews.[9] It was assumed that the elution volume, V_e, for SIF is approximately a linear function of the logarithm of the molecular weight. The Sephadex G-15 column, 25 x 400 mm, was calibrated with bacitracin (Mw 1422), 1 mg/ml, vitamin B_{12} (Mw 1355), 0.1 mg/ml, erythromycin (Mw 734), the respective solutions was used for determining the elution volume.

The eluent was 0.3% saline. The V_e-values were plotted against the logarithms of the respective molecular weights, and the molecular weight for SIF was calculated from the diagram thereby obtained.

Determination of the Streptolysin Inhibitory Factor activity in Cernitin spissum. Lyophilized streptolysin-O (prepared by the “Human” Serum Research Institute, Budapest, Hungary, for diagnostic purposes) was used as the hemolyzing enzyme. The freeze-dried content of one ampoule was dissolved in 15 ml physiological saline. (According to the manufacturers, each ampoule contains 15 test-tube doses of streptolysin-O.) The substrate used was a 2% suspension of sheep blood cells in physiological saline. The concentration of blood cells in the suspension was determined by measuring the HbO_2-content of the solution in a spectrophotometer at 576 nm after dilution 1:100 with 0.05% ammonia, using a 1 cm cuvette, against the solvent as blank standard. The OD_{576} for the diluted solution should be 0.182.

The streptolysin activity was investigated in a series of test samples according to the following.

To [0.5-0.05(*n*-1)] ml streptoylysin-O solution (*n*=dilution number) was added physiological saline to a total volume of 1.0 ml per sample. The mixtures were diluted incubated at 37°C for 30 min, after which 0.5 ml physiological phosphate buffer solution at pH 7.0, 0.2 ml physiological sodium thioglycolate solution, and 0.25 ml 2% suspension of blood cells were added to each tube. After further incubation at 37° for 30 min, the degree of hemolysis was read visually. The following gradings were used : 4 = complete hemolysis; 3 = 75% hemolysis; 2 = 50% hemolysis; 1 = 25% hemolysis; Ø = no hemolysis.

The activity of SIF in Cernitin spissum was investigated in a series of samples according to the following. To 0.5 ml of a streptolysin-O solution was added [0.5-0.05(*n*-1)] ml of a Cernitin spissum solution (*n* = dilution number), diluted 1:200 with physiological saline and saline to a total volume of 1.0 ml per sample, after which the mixtures were treated and readings taken as above. The following units were used in calculating the inhibitory activity:

(1). 1.0 streptolysin-O unit (SU) = the smallest quantity of streptolysin-O, which, under the standard conditions stated above, results in complete hemolysis of blood cells (degree of hemolysis = 4). (The concentration of streptolysin units (SU) in the streptolysin solution (1 ampoule/15 ml) being approx. 8-10 SU/ml).

(2). 1.0 Streptolysin Inhibitory Factor Unit (SIF-U) = the smallest quantity of SIF, which completely inhibits 1.0 SU under the standard conditions given above.

Investigation of the inhibitory characteristics of the Streptolysin Inhibitory Factor. (1). Cernitin spissum solution, conc. 1:200, was titrated and mixed with streptolysin solution according to the standard test method. The solutions were incubated at 37°C. The incubation times for the various series of samples were: 5, 10, 15, 20, and 30 min. The degree of inhibition in the different series was investigated and compared with the inhibition of the sample that had been incubated for 30 min. (2). Streptolysin was treated with Cernitin spissum solution containing an equivalent amount of SIF-U. The mixture was incubated at 37°C for 30 min. The streptolysin-SIF solution was dialyzed against an equal volume of physiological saline (equilibrium dialysis) in a dialysis tube of 20 mm diameter at +4°C for 24 h, after which the dialysis was allowed to continue against a large volume of saline solution for a further 24 h period at +4° C. (The volume of the outer solution was 15 times of that of the inner solution.) The SIF activity of the outer solution obtained during equilibrium dialysis was investigated and compared with the activity of untreated SIF solution that had been stored at same temperature and time. The streptolysin activity of the inner solution was investigated after 48 h dialysis and compared with the activity of untreated streptolysin that had been dialyzed under the same conditions as the treated substance, and also with streptolysin that had been stored at +4°C for 48 h.

RESULTS

Purification

Methanol fractionation. After mixing Cernitin spissum with methanol, a pale brown precipitate was obtained, having a very loose consistency. After drying, the precipitate turned dark brown. The dry weight of the precipitate was approx. 10% of the total dry weight of Cernitin spissum. The SIF activity in the extract was not reduced as a result of the methanol fractionation; all the activity was recovered in the supernatant, the sediment being completely inactive.

The OD_{257} for the supernatant was approx. 5% lower than for the untreated Cernitin spissum, the specific SIF activity [SIF-U ml^{-1} $[OD_{257}(conc.)]^{-1}$] increased by about 5% (Figs. 5, 6). (The specific activity for undiluted SIF fractions was calculated with respect to the OD_{257}, the intensity of the Folin reaction and the total content of carbohydrates.)

Gel filtration. Cernitin spissum supernatant was gel filtered on analytical scale and also on preparative scale. Gel filtration on analytical scale employed a column measuring 15 X 300

mm and sample quantities of 0.2 ml. The various saline solutions used did not differ in their separative properties. The SIF activity was recovered at the elution peak with $V_e \approx 70$ ml. The fractions which gave other UV-absorption peaks were completely inactive with respect to SIF (Fig. 2).

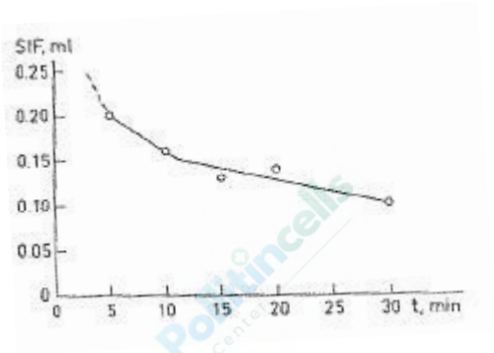

Fig. 1. Inactivation of streptolysin-O by treatment with Streptolysin Inhibitory Factor (SIF). Correlation between the quantity of SIF (ml Cernitin spissum 1:200) which inactivates 3.0 streptolysin-O units and the incubation time (t min).

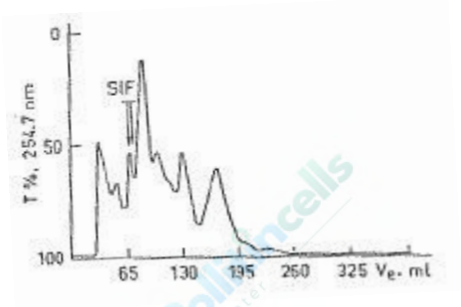

Fig. 2. Gel filtration of Cernitin spissum supernatant (1st gel filtration). Purification of Streptolysin Inhibitory Factor (SIF). Column: Sephadex G-15, 1.5x30 cm. Sample quantity: 0.2 ml. Eluent: 0.1% sodium chloride solution. Flow rate: 25 ml/h.

A number of attempts were made to concentrate the SIF active fractions: freeze-drying, vacuum drying at room temperature, and drying with cold air and hot air fans. The dried residues were then dissolved in distilled water, and the SIF activity of the solutions was investigated and compared with the activity for non-concentrated fractions. The results obtained showed that the SIF activity was completely destroyed by concentrating fractions derived from gel filtration in which 0.9% saline was used as elution fluid. The SIF activity was not affected by drying in the fractions with a 0.1% saline concentration.

Gel filtration of Cernitin spissum supernatant was also carried out on preparative scale, using a column measuring 25x400 mm. The sample quantities were 5 ml, and a 0.1% sodium chloride solution was used as eluent. The elution curve obtained showed a high absorption for the fractions. The SIF activity was recovered after 155 ml of the elution volume, and distributed within a volume of 40 ml. (The fractions between 155 ml and 195 ml were collected.) The loss of the SIF amount introduced after the first gel filtration was approx. 25%. The second gel filtration was run on a column measuring 25x400 mm, and the sample quantity was approx. 5 ml (the concentrated pool of SIF active fractions from the first gel filtration). 0.1% saline was used as eluent.

The elution diagram showed that the sample contained a few percent high molecular impurities, but no low molecular ones, after the first gel filtration. The quantity of impurities with molecular weights slightly above and below that of SIF comprised at least half the total UV absorbing substance quantity (Fig. 3).

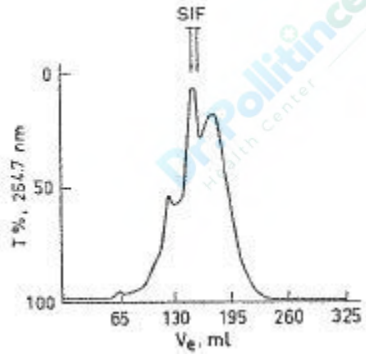

Fig. 3. Gel filtration of Cernitin spissum supernatant (2nd gel filtration). Purification of Streptolysin Inhibitory Factor (SIF). Column: Sephadex G-15, 2.5x40 cm. Sample: 5 ml concentrated pool of SIF-active fractions from 1st gel filtration (corresponding to 5 ml of Cernitin spissum supernatant). Eluent: 0.1% sodium chloride solution. Flow rate: 25 ml/h.

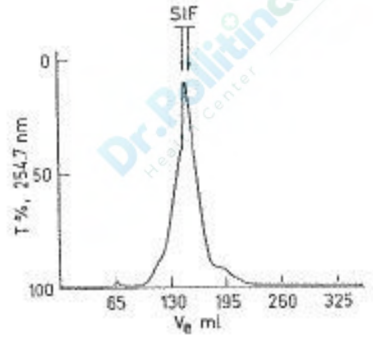

Fig. 4. Gel filtration of Cernitin spissum supernatant (3rd gel filtration). Purification of Streptolysin Inhibitory Factor (SIF). Column: Sephadex G-15, 2.5x40 cm. Sample: 3 ml concentrated pool of SIF-active fractions from 2nd gel filtration (corresponding to 5 ml of Cernitin spissum supernatant). Eluent: 0.1% sodium chloride solution. Flow rate: 25 ml/h.

The SIF activity was recovered between the elution volumes 155-195 ml. The fractions within this elution range were pooled and concentrated. In the course of the second gel filtration, a further 5% of the total SIF activity was lost.

The third gel filtration was carried out in the same way as the second. The sample volume was approx. 3 ml (concentrated pool of SIF active fractions from the second gel filtration). The elution diagram showed that the sample contained at least 5 different molecular weight groups, three of which (two with higher and one with lower molecular weight than SIF) comprised 5-7% of the total quantity of substance. The majority of the impurities, 30-40%, comprised a group with a molecular weight slightly higher than that of SIF (Fig. 4). The SIF active fractions between V_e' = 155 ml, and V_e " = 195 ml were collected. After the third gel filtration, approx. 65% of the original SIF quantity was recovered in the active fractions.

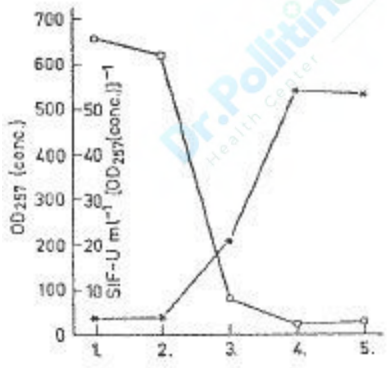

Fig. 5. Purification of Streptolysin Inhibitory Factor (SIF). Correlation between streptolysin inhibiting ativity and optical density (OD_{257}). SIF-U $[OD_{257}(conc.)]^{-1}$: ⋆; OD_{257} (conc): □. Notations on x-axis: 1. Cernitin spissum. 2. Cernitin spissum supernatant from methanol fractionation. 3. SIF-active fraction from 1st gel filtration. 4. SIF-active fraction from 2nd gel filtration. 5. SIF-active fraction from 3rd gel filtration.

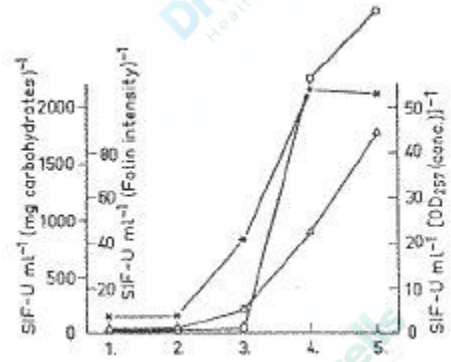

Fig. 6. Purification of Streptolysin Inhibitory Factor (SIF). Correlation between specific activity values, i.e. the streptolysin inhibiting activity in relation to the Folin intensity (SIF-U/Folin intensity: Δ), total carbohydrate content (SIF-U/carbohydrates: O), and optical density (SIF-U/OD_{257}: ⋆). Notations on x-axis: 1. Cernitin spissum. 2. Cernitin spissum supernatant from methanol fractionation. 3. SIF active fraction from 1st gel filtration. 4. SIF active fraction from 2nd gel filtration: 5. SIF active fraction from 3rd gel filtration.

Analyses of the SIF active fractions gave the results shown in Figs. 5 and 6. With respect to substances absorbing UV light at 257 nm, purification after the first gel filtration was 85-90%, after the second 95%, and after the third 98%.

As for purification wit h respect to specific SIF activity, see Table 1.

Table 1. Purification of the Streptolysin Inhibitory Factor (SIF) in pollen extract with respect to specific activity.

Degree of purity: specific SIF activity for the purified fraction
specific SIF activity for Cernitin spissum

	Degree of Purity		
	SIF-U/ml /OD_{257} conc.	SIF-U/ml/Folin Intensity	SIF-U/ml/carbohydrates
Cernitin spissum	1.0	1.0	1.0
Cernitin spissum Supernatant	1.1	1.0	1.3
1st gel filtration	5-6	4-5	2
2nd gel filtration	15	20	90
3rd gel filtration	15	40	110

Characteristics

Heat stability. SIF is resistant to heat, the activity is not reduced after incubation for 10 min at 65°C and at 100°C.

pH stability. The activity of SIF was completely destroyed after incubation for 20 min at 37°C and at a pH 2.0. At pH 11.5, approx. 60% of the SIF activity in the solution was destroyed.

Ability to dialyze. SIF is capable of dialyzing, and the activity can be quantitatively recovered in the inner and outer phases after equilibrium dialysis. The stability to dialyze is not affected by variations in the ionic strength of the solvent.

Molecular weight. The preliminary value for the molecular weight of SIF, calculated with the aid of the elution volume in gel filtration, is approx. 850. The test samples used for determining the molecular weight of SIF were Cernitin spissum supernatant and the SIF active fraction from the third gel filtration. The same results were obtained with both samples (Fig. 7).

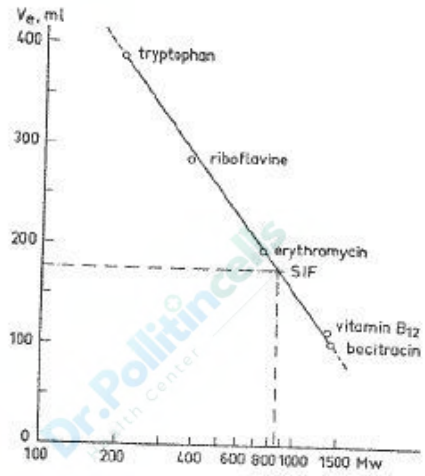

Figure 7. Determination of preliminary values for the molecular weight of the Streptolysin Inhibitory Factor (SIF), using gel filtration. Correlation between the logarithm for the molecular weight and the elution volume (V_e). Column: Sephadex G-15, 2.5X40 cm. Eluent: 0.3% sodium chloride solution.

The SIF activity of the Cernitin spissum extract. Depending on the degree of dryness (20-40%), the SIF content in Cernitin spissum varied between 1500 and 3000 SIF-U/ml.

The dependency of the degree of inhibition on the incubation time. The test results show that, when inactivating streptolysin with SIF under standard conditions, the incubation time should not be less than 20 min (Fig. 1). An incubation time of 5 min requires a SIF concentration, which is approx. 100% greater than that required in a 30 min incubation period. The difference between 15 and 30 min is approx. 30%; this value is about 20% greater than the error of the method at the SIF concentrations used in the investigation.

STREPTOLYSIN INHIBITORY FACTOR

Investigation of the activity in the inner and outer dialysis phases. This investigation with SIF-treated streptolysin gave the following results. Active SIF was recovered in the outer phase in equilibrium dialysis of SIF-treated streptolysin. No reduction in activity of SIF could be observed which could be attributed to inactivation of

streptolysin. The SIF activity in the outer phase was somewhat less than half of the original SIF activity in the inner phase inside the dialysis tube, but this halving of the concentration agreed with the dilution and was fully comparable with the results obtained in equilibrium dialysis of SIF without streptolysin. The activity of the streptolysin in the inner phase was investigated after continued dialysis against a large volume of outer phase. The results of the test showed that no streptolysin activity could be recovered after inactivation with SIF. The activity of the dialyzed streptolysin that had not been treated with SIF, diminished after 48 h dialysis by about 20% in relation to non-dialyzed streptolysin.

Storage stability. The SIF activity for Cernitin spissum solution, conc. 1:200, does not diminish during storage at room temperature or at +4°C over a period of several days. The stability of the solution is limited primarily by fungal infection, even at low temperatures. The stability of concentrated and diluted, sterile Cernitin spissum solutions is unlimited at refrigeration temperature, with respect to the SIF activity. (The streptolysin solution can be stored at +4°C for at least 48 h without suffering any loss of activity. At higher temperatures, the solution easily becomes infected. Careful handling in the presence of air does not lead to a reduction in activity.)

DISCUSSION

The Streptolysin Inhibitory Factor, SIF, is a quantitatively measurable substance occurring in pollen extract. The method described for the measurement of SIF activity is reproducible. The error of the method at lower SIF concentrations is approx. 10%, but somewhat lower at higher concentrations. The investigation had shown that SIF activity is not affected by smaller variations in incubation time or in temperature. The inhibitory activity of SIF is a linear function of the SIF concentration within a wide range.

The molecular weight of ~840 indicates that SIF is probably a condensation of a polymerization product. Other possible alternatives might be a peptide, a nucleic acid fragment, or a polysaccharide. SIF is easily soluble in water, in saline, and in 95% methanol. The fact that SIF is soluble in methanol does not eliminate any of these proposed groups of substances, since the dielectric constant of the solvent is high.

The heat stability and the stability against variations in the ionic strength of the solvent indicate a chemical structure in which the streptolysin-inhibiting activity is either reversible or resistant to these factors. The SIF activity is destroyed in association with treatment in acid environment, and partly also in alkaline environment. In view of the fact that the acid-alkali treatment takes place under mild conditions, it is hardly feasible that a component, *e.g.* acid or alkali sensitive amino acids, in SIF would thereby be destroyed.

The results obtained from the gel filtrations show that the SIF activity is probably not dependent on a co-factor. Whereas the inhibitory effect of SIF remains unchanged after its reaction with streptolysin, the latter is irreversibly inhibited, as shown by the fact that SIF can be separated from inactivated streptolysin by dialysis. The chemical analyses show that the specific SIF activity, with respect to the Folin intensity and the total carbohydrate content, increased strongly after the third gel filtration in relation to the second filtration. Concerning the optical density at 257 nm, the specific activity of the SIF active fractions was unchanged after the third gel filtration in comparison with the second filtration. This result increased the probability, that SIF might be a nucleic acid fragment.

The results of separate investigations have shown that pollen extract, produced under sterile conditions, contains a concentration of SIF which is just as high as that of the commercially produced Cernitin spissum extract.

Further studies on isolation and characterization of SIF are in progress.

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OTHER SUPPORT:

GRAMINEX Flower Pollen Extract

Therapeutic Action of a Pollen Extract

Published in Medecine Generale, France - No. 1 – May 1975

At the request of OZOTHINE Laboratories, we have studied an appetite stimulant compound marketed under the name of STENOREX, in children undergoing prolonged hospital admissions. This entirely vegetable-based product, prepared from a pollen extract, possesses neither hormonal nor antihistamine effects, but acts purely as an appetite stimulant; its principal effects are therefore exerted on weight gain and on physical and psychic asthenia.

1. Conditions of Trial

Choice of children

The trial covered 54 children chosen from three institutions: the Institution J.-B. Thiéry, the Maison d' Enfants Clairjoie, and the Pouponniére de l' Aide à l' Enfance of Meurthe-et-Moselle. The Institution J.-B. Thiéry takes children with chronic neurological ailments, mild, moderate or profound mental retardation, and children with behavior disorders or psychoses; the other two establishments take normal infants and children, for exclusively social indications.

The conditions of life of the children remained unchanged throughout the trial; in particular, diet was not altered when the trial was started. The product was only given to children who had been inmates of one of the institutions for at least four months. Children developing an acute illness in the course of the trial were excluded from it.

Dosage

In accordance with the recommendations of OZOTHINE Laboratories, we adopted the following dosage schedule:

-1 gelule daily for children weighing less than 5kg.
-2 " s " " " " from 5 to 10kg.
-3 " " " " " from 11 to 15kg.
-4 " " " " " over 16kg.

The duration of treatment was four weeks in all cases.

The product was given at mealtimes, the gelule either being swallowed with a little jam or yoghurt, or – in the case of the youngest children – the contents of the gelule being mixed with these foods.

However administered, the product was accepted with pleasure by all the children.

Age

The ages of the children ranged from 6 months to 12 years. There were 20 infants aged under 30 months, and 34 children from 2 $^{1}/_{2}$ to 12 years of age.

Indications

We accepted the following indications for treatment:

2. Plateau of weight curve. We particularly selected children whose weight curve had been stationary for several months in spite of a properly balanced diet; these children might or might not have been anorexic (see observation No. 7 and weight curve attached).

3. Severe delay in stature and weight, either in cases of long-standing constant anorexia, or – more particularly – in grossly premature infants, twins, and chronically ill children (e.g. with congenital cardiac disease)

4. Asthenic children, with low motivation either for play, food, or schoolwork. These were often children who had been hospitalized for several years, suffering from emotional deprivation and parental rejection.

A laboratory profile covering serum proteins, blood count and haemoglobin level was carried out at the beginning and end of treatment whenever this was feasible – in a total of 30 cases.

The children's weight was recorded during the month before treatment was begun, at the end of each week of treatment and finally on day 60.

2. Therapeutic Effects

Effect on appetite

Sthénorex appeared to us to have a beneficial effect upon appetite.

- In 34 out of 54 cases, the teaching or supervisory staff noted an improvement in mealtime behaviour.
- In 7 cases, there was judged to have been an excellent return of appetite.

Effect on weight curve

Remarkable weight gains were recorded in 40 out of 54 cases. The mean gain was well above that considered normal according to standard weight tables. Some cases showed spectacular gains, up to 10% or more of the initial weight.

In over half the cases, the weight gain continued during the following month.

Effect on delay in stature and weight

The duration of treatment was too short to observe any gain in stature.

Effect on asthenia

The effect of Sthénorex on behavior appeared to us to be often beneficial. Treatment improved the relationship between child and teacher; the words of encouragement when the dose was given reinforced the emotional bond that had been established; the child was consequently more cheerful, meals were awaited with pleasure and willingly eaten.

3. Tolerance and Toxicity

Tolerance of the product was perfect in every case. No digestive disturbances were recorded; there was neither vomiting nor diarrhea.

Serum protein was measured in 30 cases and a blood count done in 31.

No toxic effects were recorded; in particular there was no anemia, leucopenia or thrombocytopenia. An increase in red cell count was noted in 11 cases, and the same applies to hemoglobin levels. This facts appears to us worthy of note, in view of the presence of trace elements in the pollen extract whose administration must certainly be favourable to haemoglobin synthesis.

No sleep disturbances were noted during the trial.

The combination of Sthénorex with other medications (barbiturates, anticonvulsants,

and neuroleptic or cardiotonic drugs) did not cause any trouble.

No allergic skin reactions were noted throughout the trial.

4. Conclusion

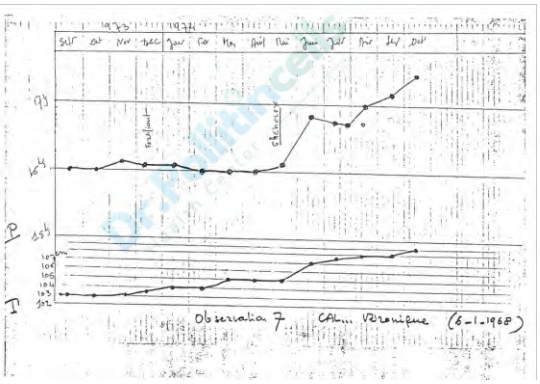
This clinical study of Sthénorex in 54 children aged from 3 months to 12 years enabled us to reach the following conclusions:

- The product is totally non-toxic.
- It is easily absorbed: it is perfectly tolerated with the digestive tract
- It has a beneficial effect on appetite and weight gain, and a significant action in correcting anaemia.
- The high success rate (19 very good results, with a weight gain of 1 kg or more, and 20 good results, with a weight gain from 400 g to 1 kg) justifies the use of the product in all cases of asthenia, anorexia or failure to gain weight.
- Finally, the fact that this is a natural product of plant origin further encourages us to recommend the use of the product.

<u>Addendum</u>

- For purposes of illustration, the weight curve of Véronique C. case No. 7, is attached; this shows an excellent weight gain after 4 months of stagnation. The effect continued during the succeeding months.

KEY TO FIGURE
Fortifiant = Tonic
P (poids) = Weight
T (taille) = Height



Pharmacological Studies of Cernilton Cernitin GBX and Cernitin T-60

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Introduction

Mixed hormones, consisting of androgens and estrogens, are commonly used for pharmacotherapy of hypertrophy.

The drug under study here is a non-hormonic preparation developed by AB Cernelle (Sweden), called CERNILTON, which is a mixture of two components, one being the oily substance Cernitin GBX and the other the water-soluble substance Cernitin T60. The two substances, contained at a ratio of 1:20, are extracted from several kinds of pollens admixed in definite proportions, after decomposition of allergens.

The subjects covered by the present study are as follows:

1. Observation of Symptoms (mice)
2. Influence on Spontaneous Movements (mice)
3. Influence on Blood Pressure and Respiration (cats)
4. Influence on Smooth Muscles (guinea pigs)
5. Diuretic Action (rats)
6. Anti-Inflammatory Action (rats)
7. Antigen-Antibody Reactions (guinea pigs)

Materials and Methods of Experiments

1. *Observation of Symptoms*

Animals were ddN strain healthy male mice with body weight of 20—25 g, each group consisting of 10 animals. The dose given was 20 times the maximum dose in human body (60 kg) both for GBX and T60 (GBX 8 mg/kg, T60 160 mg/kg). GBX was suspended in 0.1 N NaOH and 1% Tween 80 while T60 dissolved in 0.9% NaCl. Both substances were administered intravenously to the tail at a speed of 0.5 ml/min.

Observation was made as to general symptoms for 60 minutes after administration and thereafter as to both general symptoms and death occurrences.

2. *Influence on Spontaneous Movements*

Animals, were ddN strain healthy male mice with body weight of 20—25 g, each group consisting of 10 animals. Spontaneous movements were determined by means of a revolving activity

wheel with a diameter of 20 cm and a width of 5 cm, and the volume of spontaneous movements was judged by frequency of revolution. The value obtained during the 10 minutes before administration was used as the control, and the determination was carried out every 10 minutes over a period of 90 minutes after administration. Mice with values less than 70 revolutions per minute were excluded from the experiment.

GBX was suspended in 1% Tween 80 while T60 dissolved in 0.9% NaCl to make the doses 50, 100, 200 and 500 mg/kg/10 ml. The sample drugs were given by the oral route.

3. *Influence on Blood Pressure and Respiration*

After anesthetization with Pentobarbital Na at a dose of 40 mg/kg intravenously, cats (1—3 kg) were fixed in a dorsal position and changes in blood pressure recorded on smoked paper by means of a cannula and mercury mannometer previously inserted into the carotid artery; respiration was recorded simultaneously by means of a cannula and tambour inserted into the trachea.

GBX, an acidic oily substance, was alkalized to a pH of about 8.5 with 0.1 N NaOH to make intravenous administration possible. For use of the control another 0.1 N NaOH solution with the same pH was prepared. The sample drugs were administered through a vinyl tube previously inserted into the cosal vein. ECG was taken by leading from the chest both before and after administration.

4. *Influence on Smooth Muscles*

Influence was examined by means of the Magnus method on the isolated intestine, uterus and bronchus of guinea pigs and the isolated prostate of rats.

5. *Diuretic Action*

Animals were Donryu strain healthy male rats with body weight of 120—150 g bred under controlled conditions, each group consisting of 3 animals. The animals were housed separately in metabolic cages and fasted for 18 hours, and then urination was forced by finger pressure applied to the lower abdomen just before experiment. 0.9% NaCl was administered orally at a dose of 20 ml/kg while sample drugs at doses of 50, 100 and 500 mg/kg, GBX suspended in 1% Tween 80 and T60 dissolved in 0.9% NaCl. The volume of urine voided in 3 hours after administration plus that voided compulsorily was taken as the total volume of urine.

6. *Anti-Inflammatory Action*

Studies were made by means of a method using croton oil and egg albumin as inflammation-inducing substances, combined with the filter paper-pellet method. Phenylbutazone (Irgapyrine, FB) was used as the control drug.

a. *Method using croton oil and egg albumin:* Animals were Donryu strain healthy male rats with body weight of 150—200 g bred under controlled conditions (room temperature 25 ± 1° C, humidity 55 ± 5 %), each group consisting of 6 animals. The animals were employed without anesthetization. Inflammation-inducing substances used were 1% croton oil (diluted with olive oil) and 10% egg albumin; they were given at a dose of 0.1 ml/body to the rt. heel subcutaneously to induce edema. Edema-suppressing effects were judged by the swelling rate, which was obtained from measurement of swelling with a caliper 6 times (0.5, 1, 2, 3, 5, 24 hours). The sample drugs were given orally one hour before administration of the inflammation-inducing substances.

b. *Filter-paper-pellet method:* Animals were Donryu strain healthy male rats with body weight of about 130 g bred under controlled conditions (room temperature 25 ± 1° C, humidity 55 ± 5 %), each group consisting of 6 animals. After anesthetization with ether, the hair was clipped of the lumbar area and the skin incised to a length of 1—1.5 cm slightly to the right of and along the median line. Following insertion to the back site of filter paper (Toyo Roshi No. 2 cut in roud form with weight of 3 mg) sterilized with dry heat at 120° C for 40 minutes, button suture was performed and the sample drugs administered

for 4 days. The filter paper was removed 5 days after insertion and then weighed, both immediately after difference in weight was taken as the weight of the exudates and used as a guide to judgment of the degree of inflammation.

7. *Anti-gen Antibody Reaction*

a. *Sensitization by intraperitoneal administration:* Animals were divided into two groups, each consisting of 5 healthy male guinea pigs with body weight of 350—450 g bred under controlled conditions. The animals were sensitized with GBX (dissolved in 0.1 N NaOH, pH 8.5) in one group and with T60 (dissolved in 0.9% NaCl) in the other at a dose of 50 mg/body every other day 3 times in all. Three weeks after the last administration, the sample drugs were administered to a dose of 10 mg/body via the penis vein, and observation was made as to symptoms and death occurrences.

b. *Sensitization by oral administration:* As CERNILTON is given by the oral route in clinical use; sensitization was carried out through this route. Animal groups were set up in the same manner as in the case of intraperitoneal administration. Administration was carried out with T60 only, which was given at doses of 1 and 2 g/body every other day 3 times in all. Three weeks after the last administration T60 was administered via the penis vein at a dose of 30 mg/body, and observation was made as to symptoms and death occurrences.

Results of Experiments

1. *Observation of Symptoms (mice)*

a. *GBX Administration Group:* No abnormal symptoms or death occurred In the 0.1 N NaOH suspension group or in the 1% Tween 80 suspension group.

b. *T60 Administration Group:* As in the GBX administration group, abnormal symptoms or death occurred in no cases.

2. *Influence on Spontaneous Movements (mice)*

Since mice would normally show very unstable reactions in the immediate post-administration phase, only the values obtained after a lapse of 5 minutes were employed for evaluation. Value so obtained were expressed in percentage, with the control value (obtained before administration) taken as 100.

a. *GBX Administration Groups (Fig. 1):* Spontaneous movements were decreased by 25% at 15 minutes in the control group (1% Tween 80 10 ml/kg), decreasing further with lapse of time. With administration of GBX at doses of 50 and 100 mg/kg, the movements were decreased by 20—25% at 15 minutes, thereafter following a course similar to that of the control group. The 200 and 500 mg/kg groups, too, showed a similar tendency, and there was no group which registered significantly less suppression than the control group. Abnormal behavior or side-effects were not noted.

b. T60 Administration Groups (Fig. 2): Spontaneous movements were decreased by 40% at 15 minutes and by 70% at 75 minutes in the control group receiving 0.9% NaCl at a dose of 10 ml/kg. With administration of T60 at doses of 50, 200 and 500 mg/kg, the movements were decreased by 30—40 % at 15 minutes, decreasing further with lapse of time. At a dose of 100 mg/kg, the suppression was marked in the beginning as compared with the 200 and 500 mg/kg groups, but then showed a recovering tendency after 75 minutes. Generally speaking, the degree of suppression was of the same between the T60 and control groups or slightly lower in the former. No abnormal behavior or side-effects were observed.

3. *Influence on Blood Pressure and Respiration (cats)*

a. *Influence of Solvent 0.1 N NaOH (Figs. 3, 4):* With intravenous administration of 0.1 N NaOH (pH 8.5) at doses of 0.1, 0.3 and 0.5 ml/kg, the blood pressure was lowered by 1.5, 2.6 and 8.4%, respectively, after a temporary rise. The lowering, however, was also temporary. As to respiration, only slight excitement was noted in 3

of 6 cases of the 0.5 ml/kg groups. Changes in ECG were insignificant.

b. *Influence of GBX Figs. 5, 6, 7, 12):* With administration of GBX at doses of 0.1, 0.3, 0.5 and 1 mg/kg, the blood pressure was only slightly lowered (1—3%) after a temporary rise (1—3%), with no significant changes in respiration of ECG. At doses of 3, 5, 10 and 20 mg/kg, the pressure was lowered by 6, 8, 14 and 24%, respectively, after a slight temporary rise (average 0.6—2.4%), the duration being proportionate to dosage, about 5 minutes in case of the 20 mg/kg group. Influence on respiration was insignificant at doses of 3 and 5 mg/kg. At a dose of 10 mg/kg 2 of 5 cases showed a slight degree of excited respiration, while at a dose of 20 mg/kg all cases showed slight to moderate degrees of excited respiration. Electrocardiographically, only the 20 mg/kg group showed an increased heart rate (4.3%) immediately after administration. This occurred only in one of 5 cases; in the other 4 cases the rate remained unchanged or was decreased (average 5.7%).

c. *Influence of T60 (Figs. 8, 9, 10, 11, 12):* Influence was stronger with T60 than with GBX both on blood pressure and respiration. With intravenous administration in doses of 0.1, 0.3, 0.5, 1, 3, 5, 10, 20, 30 and 50 mg/kg, the blood pressure was transiently raised (0.5—5.6%), with degrees not necessarily proportionate to dosage. Subsequently, however, the pressure was lowered by 13.1, 17.5, 34.1, 43.4, 49.5, 55.3, 56.4, 62.1, 69.5 and 65.2%, showing a dose-response correlation. The lowering effect was only transient at doses of 0.1—3 mg/kg, but at higher doses (5, 10, 20, 30, 50 mg/kg) the effect was lasting, about 5 minutes at doses of 30 and 50 mg/kg, and the pressure was lowering proportionally to dosage. Effects on respiration were practically nil or extremely slight at doses of 0.1—0.5 mg/kg, but at doses of 1—20 mg/kg an excited respiration proportionate to dosage was noted. At still higher doses (30 and 50 mg/kg) the degree of excitement ranged from moderate to intense, though death due to dyspnea occurred in no cases.

Electrocardiographically, no appreciable changes were noted at doses of 5 mg/kg and below, while at doses of 10, 20, 30 and 50 mg/kg the heart rate was shown to be unsteady in the immediate post-administration phase, decreasing in some cases (2 cases, average 58%) and increasing in others (3 cases, average 19%). Changes in wave shapes were not significant.

4. *Influence on Smooth Muscles*

1) *Influence on Isolated Intestine, Uterus and Bronchus (guinea pigs)*

a. *Influence of GBX:* Though only slightly, spontaneous movements were enhanced in the smooth muscles of the intestine and uterus at concentrations of 10^{-4} g/ml and higher (final concentrations). Spastic action was not noted.

b. Influence of T60 (Figs. 13, 14, 15): At a concentration of 10^{-5} g/ml a slight degree of spasm was noted in the intestine and uterus, while at cocentrations of 10^{-4} g/l and higher a definite spastic action was noted. On the other hand, rise in tonus of the bronchial muscle was observed at a concentration of 10^{-3} g/ml.

2) *Influence on Isolated Prostate (rats)*

a. *Influence of GBX (Fig. 16):* Influence on the prostate was not revealed at all at concentrations of 10^{-3} g/ml and lower.

b. *Influence of T60 (Fig. 16):* At concentrations of 10^{-4} g/ml and lower, T60 exerted no spastic action on the prostate; but the action was noted at a concentration of 10^{-3} g/ml the degree being about the same as those observed with Ach 10^{-7}—10^{-6} g/ml and $BaCl_2$ 2 X 10^{-4}.

5. *Diuretic Action (rats)*

Sample drugs and 0.9% NaCl were given by mouth and observation was made as to the volume of urine excreted.

a. *Influence of GBX (Table 1):* With oral administration of 1% Tween 80 at a dose of 5 ml/kg (control), the urinary volume at 3 hours

was 1.46 ml on the average. With GBX at doses of 50, 100 and 500 mg/kg the volume (1.43, 1.40, and 1.26 ml) was slightly lower than the control and tended to decrease as the dosage was increased. Hence, GBX exerts no diuretic action.

b. *Influence of T60 (Table 1):* With oral administration of 0.9% NaCl (control), the urinary volume at 3 hours was 1.28 on the average. At doses of T60 50, 100, and 500 mg/kg the volume was 1.15, 1.35 and 0.70 ml, with no diuretic action.

6. Anti-Inflammatory Action (rats)

1) *Effects on Croton Oil-Induced Edema*

a. *Croton oil-induced edema (Table 2):* Edema due to croton oil varied little in terms of swelling rate up to 5 hours. Thereafter, it increased with time, showing an increase of 50—55% at 24 hours.

b. Effects of GBX (Table 2): Suppressive effects on croton oil-induced edema were not observed at all from 0.5 to 2 hours at oral doses of GBX 100, 200 and 500 mg/kg. At doses of 100 and 500 mg/kg the swelling rate was increased with time, whereas at a dose of 200 mg/kg the rate was decreased by 6, 7 and 12% at 3, 5 an 24 hours over the control, with significant difference at a risk rate of 5% at 24 hours (Table 10).

c. *Effects of T60 (Table 3):* Suppressive effects on croton oil-induced edema were not noted at all with T60 at a dose of 100 mg/kg. At doses of 200 and 500 mg/kg edema was suppressed by 14 and 7% at 0.5 hour, 12 and 9% at 1 hour, 6 and 4% at 3 hours, 6 and 2% at 5 hours, and 7% at 24 hours (500 mg/kg group only) over the control, with significant difference at a risk rate of 5% between the 200 mg/kg and control groups at 0.5 and 1 hour (Table 10).

2) *Effects of Albumin-Induced Edema*

a. *Effects of GBX (Table 4):* Albumin-induced edema was suppressed by 9% at 5 hours at a dose of 100 mg/kg and by 11% at 0.5 hour at a dose of 500 mg/kg over the control, but no significant difference was noted at either dose. At a dose of 200 mg/kg suppressive effects were scarcely noted up to 5 hours. On the other hand, at 24 hours, all groups showed a suppression of 9—10% with significant difference at a risk rate of 1% against the control (Table 10).

b. *Effects of T60 (Table 5):* With administration of T60 at doses of 100, 200 and 500 mg/kg, the swelling rate tended to increase with dosage. Hence, T60 exerts no suppressive action on albumin-induced edema.

3) *Effects Observed by Means of Filter-Paper-Pellet Method*

a. *Effects of GBX (Table 6):* With oral administration of 1% Tween 80 at a dose of 5 ml/kg (control), the weight of granuloma was 163.2 mg on the average. With administration of GBX, the weight tended to decrease as the dosage was increased, the values being 83.1, 80.7 and 74.9 mg for the 100, 200 and 500 mg/kg groups, respectively, or 50.9, 49.4 and 45.9%, taking the control value as 100. No significant difference, however, was noted between these and the control groups at a risk rate of 5%.

The average weight of dry granuloma (150°C, 40 minutes) was 20.0, 10.8, 13.8 and 12.6 mg for the control, 100, 200 and 500 mg/kg groups, respectively. Expressed in percentage, the values were 100, 54.0, 69.0 and 63.0%, with the control taken as 100.

b. *Effects of T60 (Table 7):* The average weight of granuloma on oral administration of 0.9% NaCl at a dose of 5 ml/kg (control) was 139.2 mg. On the other hand, with administration of T60 at doses of 50, 100, 200 and 500 mg/kg, the weight was 73.3, 40.2, 46.3 and 73.8 mg, or 52.7, 28.9, 33.3 and 53.0%, taking the control as 100. While the difference was insignificant between the control and the 50 and 500 mg/kg groups, it was significant at a risk of 5% between the control and the 100 and 200 mg/kg groups (Table 11).

The weight of dry granuloma was 20.5 mg for the control and 11.0, 6.5, 9.1 and 12.1 mg for the 50, 100 and 500 mg/kg groups, i.e. 53.7, 31.7, 44, 4 and 59.0%, taking the control as 100. The values were relatively low in the 100 and 200 mg/kg groups.

c. *Effects of GBX + T60* (Table 8): GBX and T60 were mixed at a ratio of 1:1 and given orally. At doses of 25 mg + 25 mg, 50 mg + 50 mg, 100 mg + 100 mg, 200 mg + 200 mg and 500 mg + 500 mg, the average weight of granuloma was 105.2, 87.3, 77.3, 105.0 and 110.5 mg, while the control (1% Tween 80 5 ml/kg) was 163.2 mg. Taking the control as 100, the values were then 64.5, 53.5, 47.4 and 66.7%, with no significant difference between the control and experimental groups.

The average weight of dry granuloma was 20.0 mg for the control group and 16.0, 11.3, 11.5, 13.5 nd 15.6 mg for the experimental groups, or 80.0, 56.5, 57.5, 67.5 and 78.0%, taking the control as 100.

d. *Effects of Phenylbutazone (PB) (Table 9):* With oral administration of PB at doses of 100 and 200 mg, the average weight of granuloma was 86.2 mg (average of 5 cases) and 62.6 mg. Taking the control as 100 (163.2 mg: average of 6 cases), the values were then 52.8 and 38.4, respectively, with significant difference at a risk rate of 5% (Table 10).

The average weight of dry granuloma, on the other hand, was 20.0 mg for the control group and 17.6 and 20.3 mg for the PB 100 and 200 mg/kg groups, or 88.0 and 101.5%, respectively.

The anti-inflammatory effect was approximately of the same degree between the T60 and GBX T60 groups and the PB groups, or slightly higher in the former groups. Toxicity was higher with PB. With PB at doses of 100 and 200 mg/kg, death occurred in one out of 6 cases in each dose group, while with GBX, T60 and GBX + T60 there were no such occurrences.

e. *Weight of exudates (Table 11, Fig. 17):* The difference in weight between granuloma and dry granuloma was taken as the weight of exudate and used as a guide to judgment of anti-inflammatory effects.

With GBX at doses of 200 and 500 mg/kg, the values were lower than the control, though the difference was insignificant. With T60 at doses of 200 and 500 mg/kg and PB at a dose of 200 mg/kg, the difference was significant at a risk of 10% against the control.

7. *Antigen-Antibody Reactions (guinea pigs)*

1) *Sensitization by Intraperitoneal Route*

a. *Sensitization with GBX:* Animals were allowed to assume free positions immediately after provocative administration, but not abnormal symptoms were observed and death occurred in no cases even after 24 hours.

b. *Sensitization with T60:* One out of 5 cases died on the 18th day after commencement of the experiment, and therefore observation was made only in 4 cases. All 4 cases showed intermittent coughing 1—4 times from about one minute after provocative administration. One case developed persistent dyspnea; it gradually weakened and eventually died after 24 hours.

In case of albumin shock, death usually occurs in 2—3 minutes. Since with T60 a longer time was required, and since a sudden lowering of pressure was noted on intravenous administration of T60, it is difficult to say that the death was due to shock. Nevertheless, a slight shock symptom was clearly observed.

2) *Sensitization by Oral Route*

At doses of 1 and 2 g/body there were observed no abnormal symptoms or only slight coughing, and death occurred in no cases.

Summary

Results obtained above may be summarized as follows.

1. With intravenous administration of GBX and T60 at a dose 20 times the maximum dose in

human body (60 kg), there occurred neither abnormal symptoms nor death in mice.

2. With oral administration of GBX and T60 at doses of 50, 100, 200 and 500 mg/kg, spontaneous movements, as determined in mice by means of a revolving activity wheel, showed no suppression. Abnormal behavior was not observed, either.

3. With intravenous administration of GBX at doses of 0.1—1.0 mg/kg, the influence on blood pressure was only slight, while at doses of 3, 5, 10 and 20 mg/kg there occurred a pressure lowering proportionate to dosage (6—24%) after a transient rise (average 0.6—2.4%). The effect was lasting, about 5 minutes at a dose of 20 mg/kg. With intravenous administration of T60 at doses of 0.1, 0.3, 0.5, 1, 3, 5, 10, 20, 30 and 50 mg/kg, the blood pressure lowered, after a transient rise (average 0.5—5.6%), by 13.1, 17.5, 34.1, 43.4, 49.5, 55.3, 56.4, 62.1, 69.5 and 65.2%. There was noted a correlation between the pressure lowering and excited respiration. The effect was transient at doses of 3 mg/kg and below while lasting (proportionate to dosage) at higher doses, about 5 minutes at doses of 30 and 50 mg/kg.

4. With intravenous administration of GBX at a dose of 10 mg/kg, a slight degree of excited respiration was noted. At a dose of 20 mg/kg the degree ranged from slight to moderate.

5. With intravenous administration of T60, respiration was slightly excited at doses of 0.5 mg/kg and below. The degree, however, increased with dosage, ranging from moderate to intense at doses of 30 and 50 mg/kg, through death due to dyspnea occurred in no cases.

6. ECG changes were not marked with GBX. With T60 at doses of 10—50 mg/kg, bradycardia (58% at 50 mg/kg) or tachycardia (19% at 50 mg/kg) was noted immediately after administration. No marked changes, however, were noted in the wave shapes.

7. With GBX at a high concentration (10^{-4} g/ml), spontaneous movements of the intestine and uterus were enhanced in guinea pigs; spasms were not caused. With T60 enhanced spontaneous movements of slight spasm was noted at a concentration of 10^{-5} g/ml, and at a high concentration of 10^{-4} g/ml the tonus was definitely increased. The tonus of the bronchus was increased only at an extremely high concentration of 10^{-3} g/ml (T60). The isolated prostate of rats showed increased tonus only at a high concentration of T60 (10^{-3} g/ml).

8. At dosesof 500 mg/kg and below, oral administration of GBX and T60 exerted scarce diuretic action in rats.

9. Croton oil-induced edema was not suppressed with GBX at doses of 100, 200 and 500 mg/kg up to 5 hours after administration. Suppression was noted only at a dose of 200 mg/kg at 24 hours. T60, too, showed suppressive effects only at a dose of 200 mg/kg, with significant difference against the control at 0.5 and 1 hour.

Albumin-induced edema was suppressed at 24 hours with GBX at doses of 100, 200 and 500 mg/kg. The effect, however, was not observed with T60.

10. While by means of the filter-paper-pellet method anti-inflammatory effect was not revealed with GBX, it was noted with T60 at doses of 100 and 200 mg/kg. With GBX + T60 mixed at a ratio of 1:1, the effect was not observed.

Anti-inflammatory effect was approximately of the same degree between the T60 and GBX + T60 groups and the FB group, or slightly higher in the former groups. Toxicity was lower also in the former groups.

11. Anaphylaxis did not occur with GBX. With T60 coughing occurred in all 4 cases, one of which died of dyspnea subsequently. Sensitization by the oral route induced no specific abnormal symptoms.

Discussion

What may give rise to questions is the lowering of blood pressure and anaphylaxis seen with T60 and, possible, the action on smooth muscles. The central depressing action was not revealed.

While diuretic action was not noted here, in another experiment the urinary volume was slightly increased after prolonged administration.

Suppressive action on croton oil-induced edema was noted with GBX and T60 at a dose of 200 mg/kg, the former at 24 hours and the latter at one hour after administration. On the other hand, suppressive action on albumin-induced edema was noted at 24 hours with GBX. By means of the filter-paper-pellet method, suppressive action was noted with T60, the degree being about the same as that with Phenylbutazone while the toxicity being much lower. This point may well be included in the mechanism of action of this drug in the treatment of prostatitis and prostatic hypertrophy since, as revealed in our earlier study on its subacute and chronic toxicity, the drug can reduce the weight of the prostate without affecting generation of sperms even at small doses.

With intravenous administration of this drug at a dose of 10 mg/kg, the blood pressure may be lowered by about 50 mm Hg, due possibly to the 1% content of potassium in T60. In practice, however, such problem would not occur since clinically the drug is administered by the oral route.

Although GBX will not induce anaphylactic shock by itself, it may cause a mild degree of anaphylaxis at a probability of 25%, if given intravenously at a dose of 10 mg/kg after sensitization with T60 subcutaneously. This danger, however, is extremely remote since the drug produces no abnormal symptoms by the oral route and furthermore it is already confirmed as having practically no antigenicity or sensitinogenicity (Kimura et al., Bacteriological Dept., Nippon Medical College, “Immuno-Serological Studies of Cernitin GBX and Cernitin T60”).

Influence on smooth muscles occurs only at high concentrations of T60 (10^{-5} g/ml and higher), which may also be due to the presence of 1% potassium in T60.

Conclusions

1. With intravenous administration of GBX and T60 at a dose of 20 times as much as the maximum dose in human body, there occurred no abnormal symptoms in mice. Neither was influence noted on spontaneous movements in mice with oral administration of GBX at a dose 63 times of T60 60 times as much as the maximum dose in human body.
2. The blood pressure was lowered proportionally to dosage after a transient rise both with GBX and T60. The degree of lowering, however, was greater with T60, by 6—24% with GBX at doses of 3—20 mg while by 13.1—69.5% with T60. The pressure was lowered for 5 minutes. Bradycardia and tachycardia were noted with T60, but ECG was not markedly changed.
3. Even on smooth muscles the influence was greater with T60. While spontaneous movements of the intestine were enhanced in guinea pigs with GBX at high concentrations, with T60 spasm occurred and the bronchial muscle and prostate increased in tonus.
4. Diuretic action was not observed in rats, though observable by prolonged administration.
5. Croton oil-induced edema was suppressed at a dose of 200 mg/kg both with GBX and T60, the former at 24 hours and the latter at one hour after administration. Suppression of albumin-induced edema was noted with GBX, while by means of the filter paper-pellet method suppressive action was noted with T60.
6. The risk of anaphylactic shock was about 25% in guinea pigs. The danger, however, is extremely remote since the drug causes no abnormal symptoms by the oral route and since immunologically it is confirmed as having practically no antigenicity or sensitinogenicty.

Table 1. Diuretic Action of GBX and T60

After Premedication with 0.9% NaCl 20 ml/kg

Sample Drugs	Body Weight	Urinary Volume 0-3 hrs.	Average
1% Tween 80 5 ml/kg P.O.	124 130 158	1.50 1.45 1.42	1.46
Cernitin GBX 50 mg/kg P.O.	130 133 113	1.40 1.45 1.45	1.43
Cernitin GBX 100 mg/kg P.O.	130 130 135	1.60 1.35 1.25	1.40
Cernitin GBX 500 mg/kg P.O.	140 121 112	1.62 1.00 1.15	1.26
0.9% NaCl 5 ml/mg P.O.	136 124 134	1.20 1.35 1.30	1.28
Cernitin T60 50 mg/kg P.O.	124 140 130	1.45 0.76 1.25	1.15
Cernitin T60 100 mg/kg P.O.	129 136 124	0.90 1.60 1.54	1.35
Cernitin T60 500 mg/kg P.O.	132 152 128	0.65 0.4 0.80	0.70

Table 2. Effects of GBX on Croton Oil – Induced Edema (rats)

Drug/Time (hr)	1% Tween 80 5 ml/kg P.O	Cernitin GBX (mg/kg P.O)		
		100	200	500
Control	4.70 ± 0.18	4.80 ± 0.18	4.79 ± 0.18	4.51 ± 0.52
0.5	6.33 ± 0.45	6.44 ± 0.42	6.43 ± 0.21	6.58 ± 0.50
1	6.33 ± 0.49	6.64 ± 0.53	6.43 ± 0.28	6.48 ± 0.71
2	6.20 ± 0.40	6.59 ± 0.56	6.45 ± 0.06	6.42 ± 0.56
3	6.48 ± 0.57	6.59 ± 0.57	6.32 ± 0.42	6.31 ± 0.37
5	6.68 ± 0.49	6.90 ± 0.60	6.47 ± 0.51	6.51 ± 0.37
24	7.29 ± 0.40	7.38 ± 0.42	6.84 ± 0.28	17.18 ± 0.41

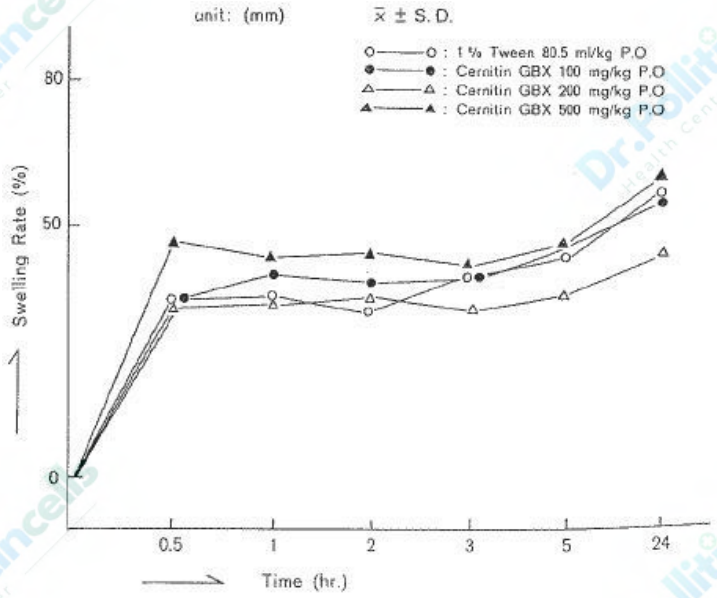


Table 3. Effects of T60 on Croton Oil – Induced Edema (rats)

Drug/Time (hr)	0.9% NaCl 5 ml/kg P.O	Cernitin T60 (mg/kg P.O)		
		100	200	500
Control	4.67 ± 0.22	4.67 ± 0.29	4.76 ± 0.15	4.60 ± 0.19
0.5	6.74 ± 0.28	6.68 ± 0.24	6.27 ± 0.47	6.33 ± 0.35
1	6.83 ± 0.36	6.74 ± 0.21	6.41 ± 0.28	6.36 ± 0.20
2	6.57 ± 0.31	6.58 ± 0.21	6.32 ± 0.27	6.20 ± 0.26
3	6.50 ± 0.29	6.48 ± 0.28	6.37 ± 0.29	6.23 ± 0.20
5	6.48 ± 0.26	6.63 ± 0.26	6.35 ± 0.34	6.34 ± 0.26
24	7.14 ± 0.49	7.10 ± 0.41	7.39 ± 0.09	6.75 ± 0.66

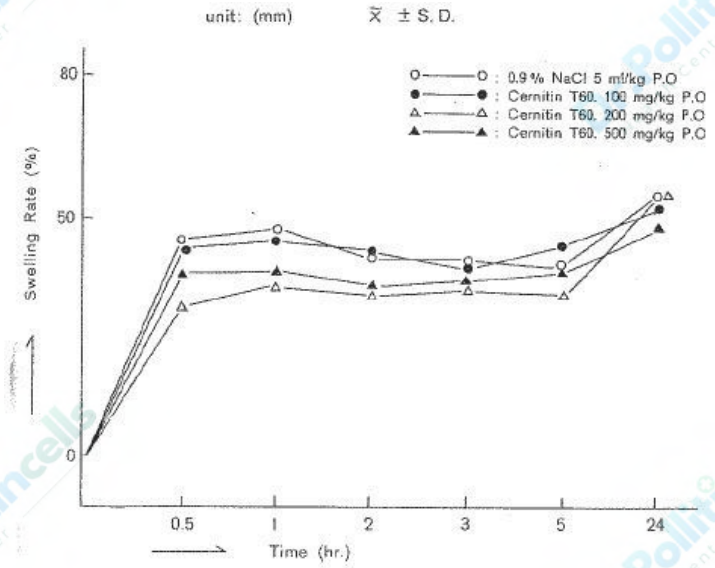


Table 4. Effects of GBX on Albumin – Induced Edema (rats)

Drug/Time (hr)	1% Tween 80 5 ml/kg P.O	Cernitin GBX (mg/kg P.O)		
		100	200	500
Control	4.55 ± 0.15	4.80 ± 0.09	4.73 ± 0.09	4.70 ± 0.28
0.5	7.21 ± 0.71	7.52 ± 0.26	7.39 ± 0.15	6.98 ± 0.89
1	7.12 ± 0.29	7.28 ± 0.34	7.27 ± 0.40	7.09 ± 0.78
2	7.08 ± 0.15	7.20 ± 0.51	7.27 ± 0.38	7.05 ± 0.55
3	7.03 ± 0.32	7.16 ± 0.39	7.25 ± 0.30	7.27 ± 0.50
5	6.96 ± 0.27	6.92 ± 0.46	7.11 ± 0.26	6.98 ± 0.61
24	5.70 ± 0.13	5.50 ± 0.25	5.46 ± 0.23	5.42 ± 0.30

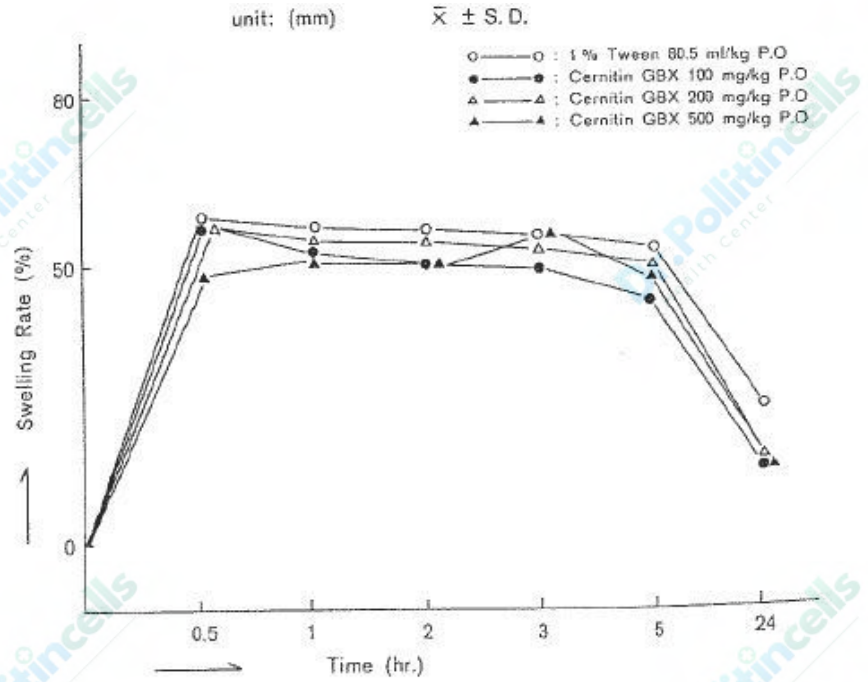


Table 5. Effects of T60 on Albumin – Induced Edema (rats)

Drug/Time (hr)	0.9% NaCl 5 ml/kg P.O	Cernitin GBX (mg/kg P.O)		
		100	200	500
Control	4.88 ± 0.26	4.74 ± 0.08	4.53 ± 0.25	4.55 ± 0.33
0.5	7.43 ± 0.52	7.24 ± 0.69	6.96 ± 0.68	7.06 ± 0.74
1	7.31 ± 0.31	7.08 ± 0.30	7.21 ± 0.43	7.26 ± 0.69
2	7.42 ± 0.34	7.07 ± 0.29	7.18 ± 0.47	7.28 ± 0.50
3	7.09 ± 0.41	7.04 ± 0.40	7.07 ± 0.55	7.18 ± 0.38
5	6.82 ± 0.32	6.76 ± 0.35	6.84 ± 0.37	6.82 ± 0.41
24	5.17 ± 0.16	5.28 ± 0.31	5.51 ± 0.42	5.71 ± 0.42

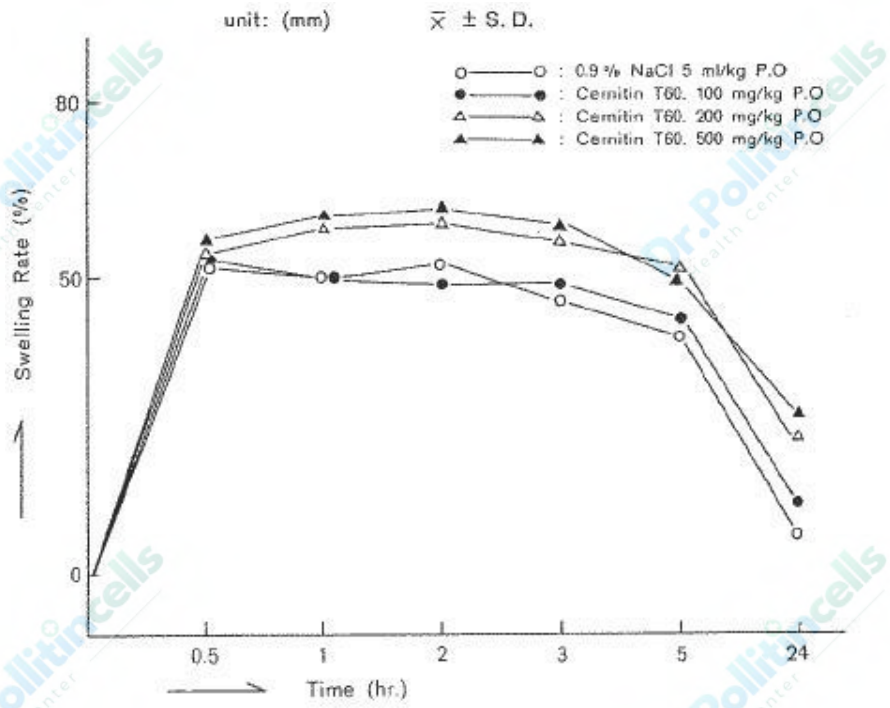


Table 6. Anti-Inflammatory Action of GBX by Means of Filter Paper-Pellet Method (rats)

NO	Granuloma				Dry Granuloma			
	1 % Tween 80 5 ml/kg P.O	Cernitin GBX (mg/kg P.O)			1% Tween 80 5 ml/kg P.O	Cernitin G BX (mg/kg P.O)		
		100	200	500		100	200	500
1	57.5	34.0	128.5	68.5	12.0	5.5	22.5	12.5
2	193.5	146.5	65.0	70.0	22.5	15.0	10.5	12.0
3	348.5	42.0	43.0	57.5	40.5	7.0	9.5	11.0
4	57.5	75.5	24.5	114.0	9.0	11.5	6.5	17.5
5	96.0	134.5	86.5	105.0	12.5	17.0	14.0	16.5
6	226.0	166.0	136.0	34.5	23.5	8.5	19.5	6.0
$\bar{X}$ ± S. E	163.2 ± 46.9	88.4 ± 19.2	80.7 ± 18.4	74.9 ± 12.2	20.0 ± 4.8	10.8 ± 1.9	13.8 ± 2.5	12.6 ± 1.7

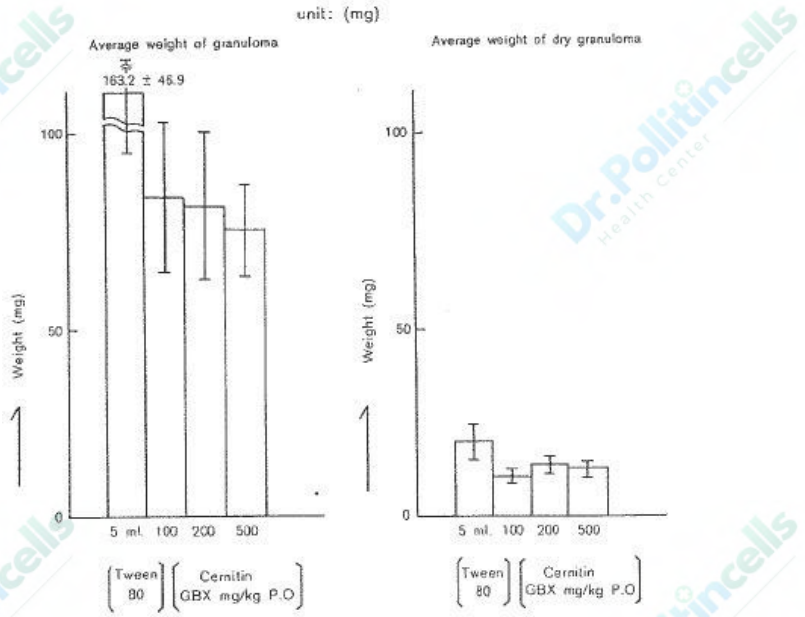


Table 7. Anti-Inflammatory Action of T60 by Means of Filter Paper-Pellet Method (rats)

NO	Granuloma					Dry Granuloma				
	0.9% NaCl 5 ml/kg P.O	Cernitin T60 (mg/kg P.O)				0.9% NaCl 5 ml/kg P.O	Cernitin T60 (mg/kg P.O)			
		50	100	200	500		50	100	200	500
1	288.5	66.0	41.0	95.5	46.0	37.0	8.5	7.0	17.0	8.5
2	77.0	63.0	31.5	31.0	73.5	11.0	11.5	5.5	7.5	12.5
3	67.0	86.0	25.0	21.0	71.0	9.5	11.5	4.5	5.0	11.5
4	299.5	73.5	44.0	51.0	63.5	47.0	11.0	6.5	10.0	11.0
5	53.5	75.0	46.0	37.5	82.0	8.5	12.0	70	7.0	12.0
6	77.5	76.5	53.5	41.5	106.5	14.5	11.5	8.5	8.0	17.0
7	111.5					16.0				
$\bar{X}$ ± S. E	139 ± 40.5	73.3 ± 3.3	40.2 ± 4.2	46.3 ± 10.7	73.8 ± 8.2	20.5 ± 5.7	11.0 ± 0.5	6.5 ± 0.6	9.1 ± 1.7	12.1 ± 1.1

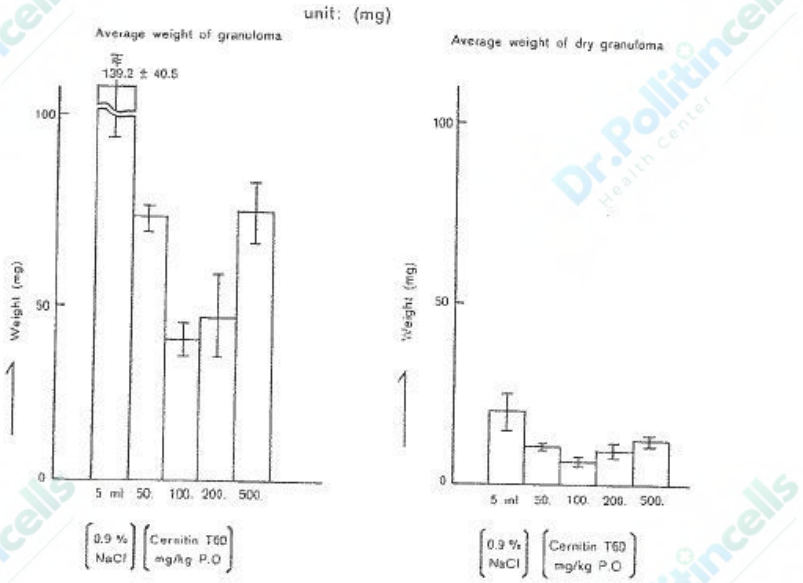
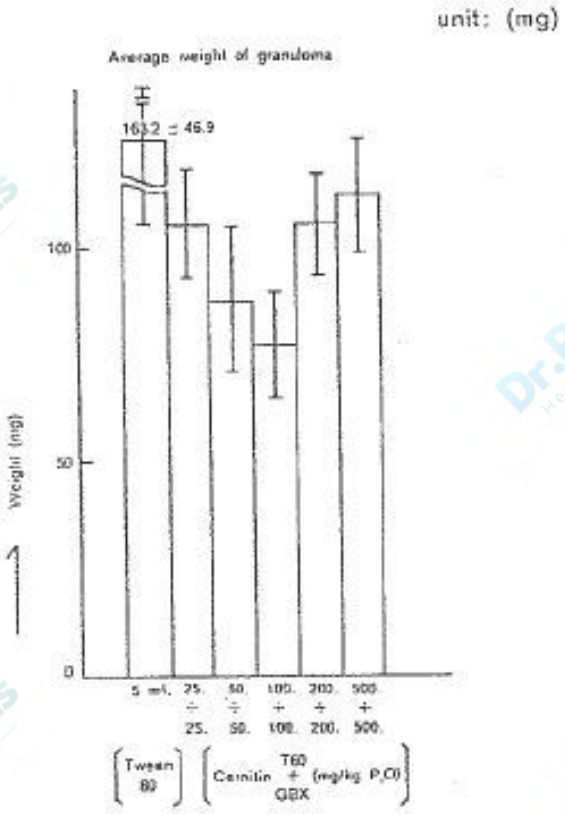


Table 8. Anti-Inflammatory Action of T60 + GBX

NO	Granuloma					
	1% Tween 80 5 ml/kg P.O	Cernitin T60 + GBX (mg/kg P.O)				
		25 + 25	50 + 50	100 + 100	200 + 200	500 + 500
1	57.5	134.0	53.0	30.0	64.0	102.0
2	193.5	64.0	57.0	64.0	76.0	49.0
3	348.5	90.0	45.5	114.5	130.5	121.0
4	57.5	111.0	139.5	97.0	116.5	106.0
5	96.0	85.0	122.5	80.5	139.0	146.0
6	226.0	147.0	106.5	77.5	104.0	139.0
$\bar{X}$ ± S. E	163.2 ± 46.9	105.2 ± 12.8	87.3 ± 16.5	77.3 ± 11.8	105.0 ± 12.2	110.5 ± 14.2



by Means of Filter Paper-Pellet Method (rats)

NO	Dry Granuloma					
	1% Tween 80 5 ml/kg P.O	Cernitin T60 + GBX (mg/kg P.O)				
		25 + 25	50 + 50	100 + 100	200 + 200	500 + 500
1	12.0	22.0	6.5	4.0	9.5	16.0
2	22.5	10.5	8.5	11.0	11.0	8.0
3	40.5	13.5	8.0	14.0	16.5	16.0
4	9.0	17.5	15.0	12.0	16.0	15.5
5	12.5	13.5	18.5	14.5	15.0	19.0
6	23.5	19.0	11.0	14.0	13.0	19.0
$\bar{X}$ ± S. E	20.0 ± 4.8	16.0 ± 1.7	11.3 ± 1.9	11.5 ± 1.6	13.5 ± 1.2	15.6 ± 1.7

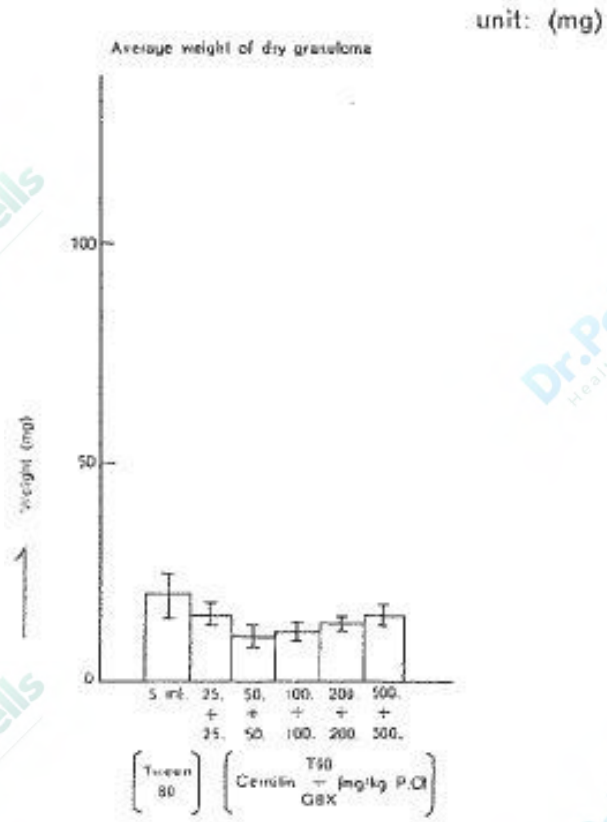


Table 9. Anti-Inflammatory Actino of Irgapyrine by Means of Filter Paper-Pellet Method (rats)

NO	Granuloma		Dry Granuloma	
	Irgapyrin mg/kg P.O		Irgapyrin mg/kg P.O	
	100	200	100	200
1	Death	64.5		13.0
2	67.0	Death	10.0	
3	160.0	59.0	34.5	16.0
4	70.0	63.0	14.5	35.0
5	85.5	72.0	14.5	17.0
6	48.5	54.5	14.5	20.5
$\bar{X}$ ± S. E	86.2 ± 19.4	62.6 ± 2.9	17.6 ± 4.3	20.3 ± 3.9

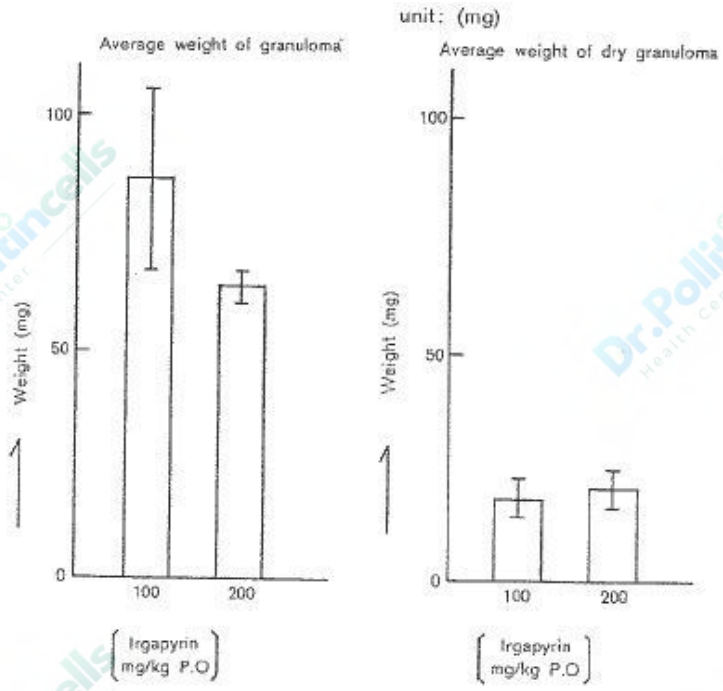


Table 10. Anti-Inflammatory Action of GBX and T60 and GBX (Summary)

Sample Drugs	Dosage (mg/kg)	Croton Oil						Egg albumin						Filter Paper-pellet method		
		0.5 h	1	2	3	5	24	0.5 h	1	2	3	5	24	5	⊟	⊟
GBX	100	—	—	—	—	—	—	—	—	—	—	—	+**	—		
	200	—	—	—	—	—	+*	—	—	—	—	—	+**	—		
	500	—	—	—	—	—	—	—	—	—	—	—	+**	—		
T-60	50	—	—	—	—	—	—	—	—	—	—	—	—	—		
	100	—	—	—	—	—	—	—	—	—	—	—	—	+*		
	200	+*	+*	—	—	—	—	—	—	—	—	—	—	+*		
	500	—	—	—	—	—	—	—	—	—	—	—	—	—		
GBX + T-60	25+25													—		
	50+50													—		
	100+100													—		
	200+200													—		
	500+500													—		
Irgapyrine	100													—		
	200													+*		

— ineffective + effective +* : with significant difference against control at 0.05>p
+**: with significant difference against control at 0.01>p

Table 11. Weight of Exudate
(Granuloma minus Dry-granuloma)

Sample / Dose	Control	50 mg/kg (P.O.)	100 mg/kg (P.O.)	200 mg/kg (P.O.)	500 mg/kg (P.O.)	
GBX	143.2 ± 42.2 (100)		88.9 ± 69.5 (62.1)	66.8 ± 16.0 (46.6)	64.0 ± 8.3 (44.7)	
T-60	118.2 ± 34.9 (100)	62.3 ± 3.1 (52.5)	33.7 ± 3.7* (28.4)	37.2 ± 9.0* (31.3)	61.7 ± 7.1 (52.0)	
Irgapyrine	143.2 ± 42.2 (100)		68.6 ± 15.4 (47.9)	42.3 ± 5.1* (29.5)		
Sample / Dose	Control	25 + 25	50 + 50	100 + 100	200 + 200	500 + 500
GBX + T-60	143.2 ± 42.2 (100)	89.2 ± 11.2 (62.3)	76.1 ± 15.0 (53.1)	65.7 ± 10.5 (45.9)	91.5 ± 11.1 (64.0)	95.0 ± 12.7 (66.3)

Control { GBX / Irgapyrine / GBX + T-60 } 1 % Tween 80

T-60 : 0.9 % NaCl

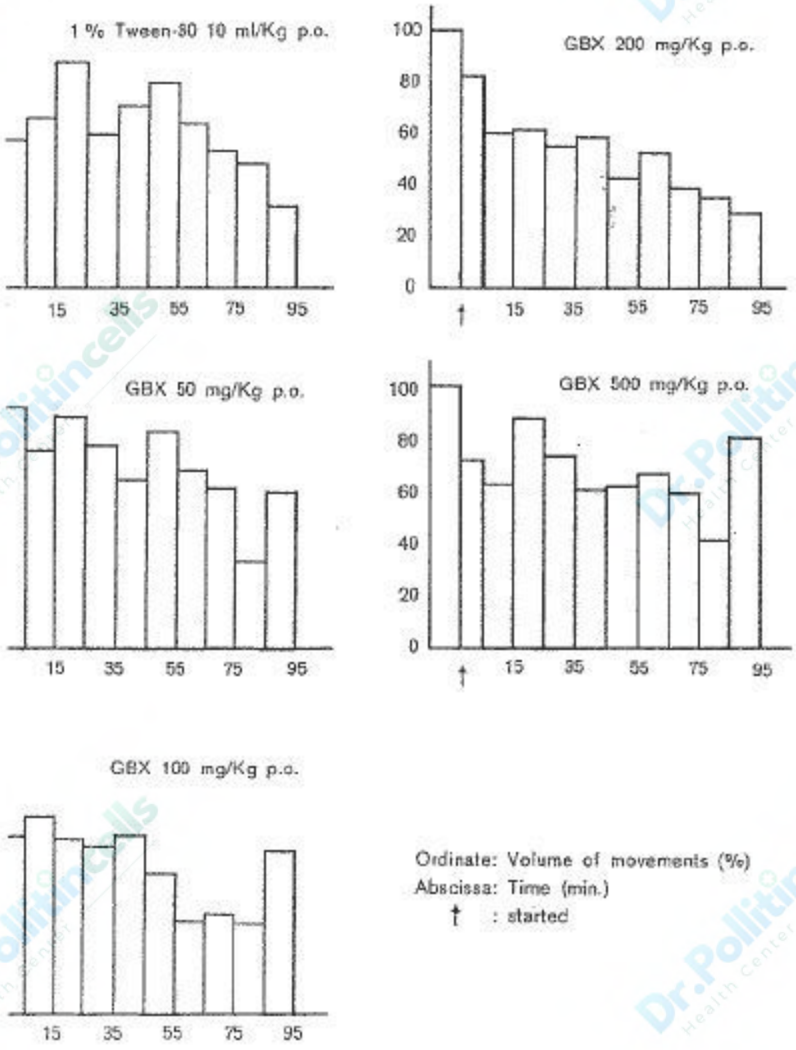
( ) : %

* : 0.1 > P (Effective)

Mean ± S.E.

Volume of Spontaneous Movements After Administration of GBX (mice)

(Before Administration = 100)



Ordinate: Volume of movements (%)
Abscissa: Time (min.)
† : started

Fig. 2. Volume of Spontaneous Movements after Administration of T60 (mice)

(Before Administration = 100)

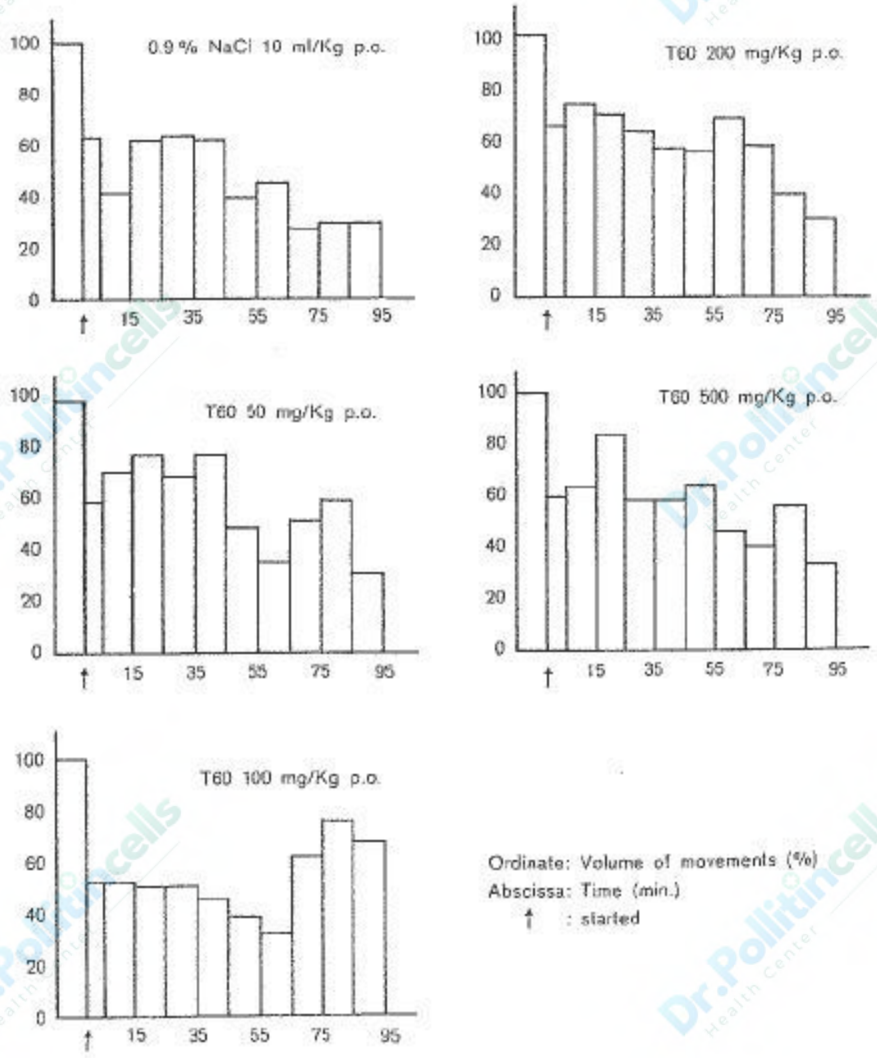


Fig. 3. Influence of Cernilton on Blood Pressure and Respiration (cat).

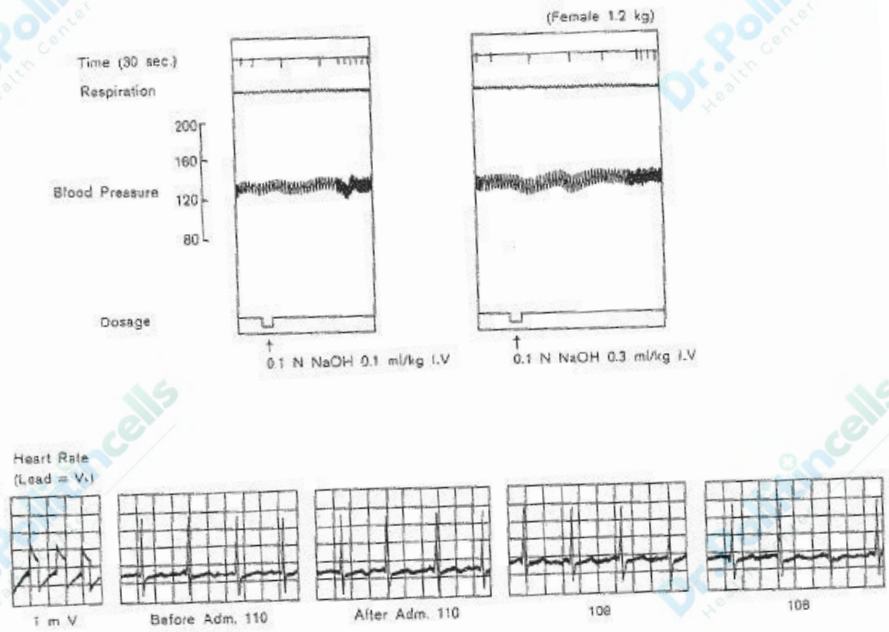


Fig. 4. Influence of Cernilton on Blood Pressure and Respiration (cat).

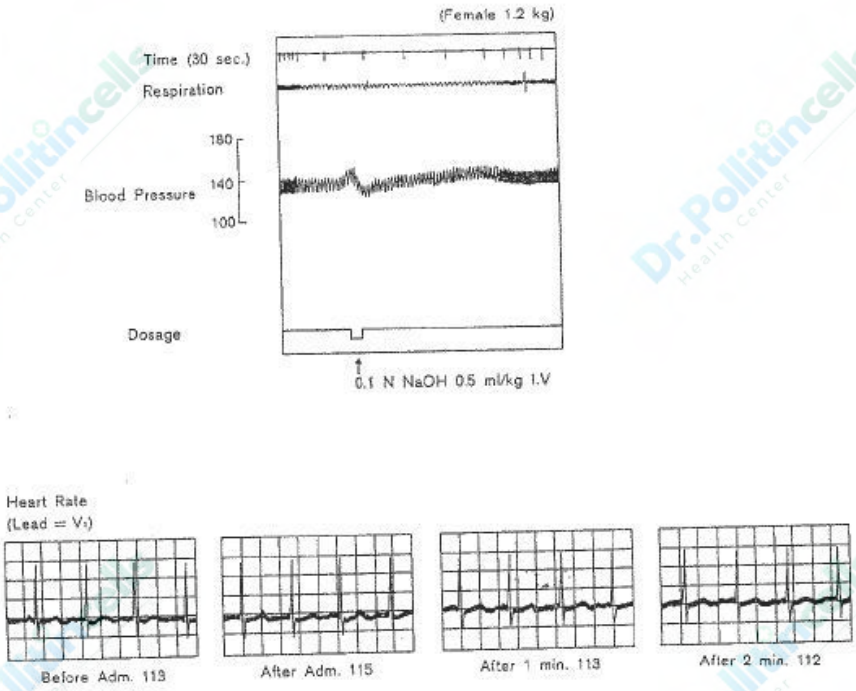


Fig. 5. Influence of Cernilton on Blood Pressure and Respiration (cat).

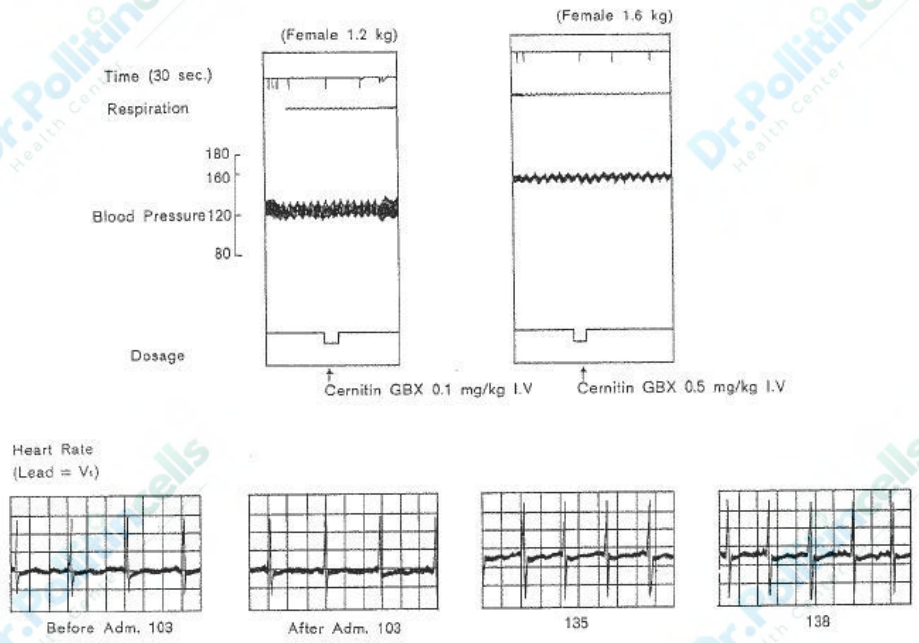


Fig. 6. Influence of Cernilton on Blood Pressure and Respiration (cat).

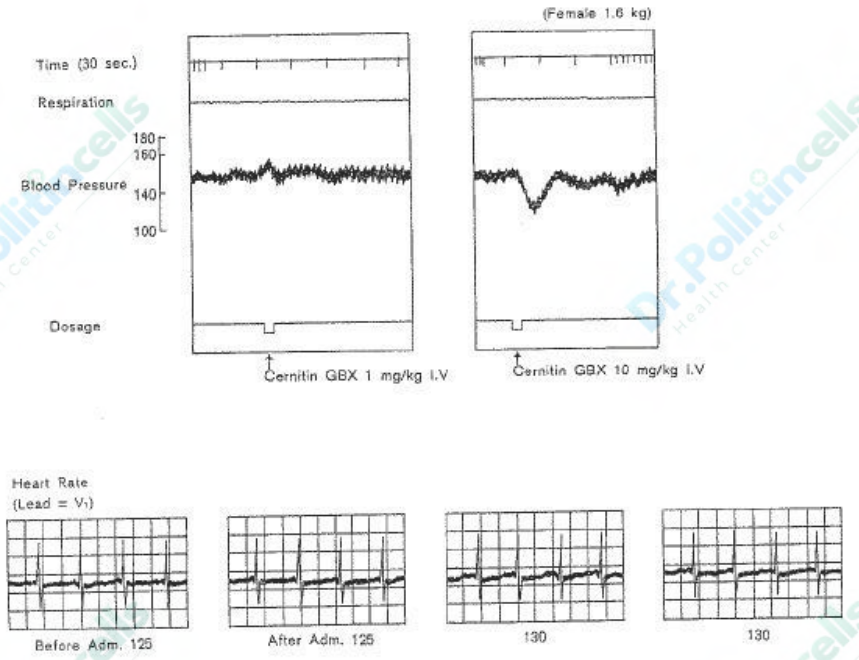


Fig. 7. Influence of Cernilton on Blood Pressure and Respiration (cat).

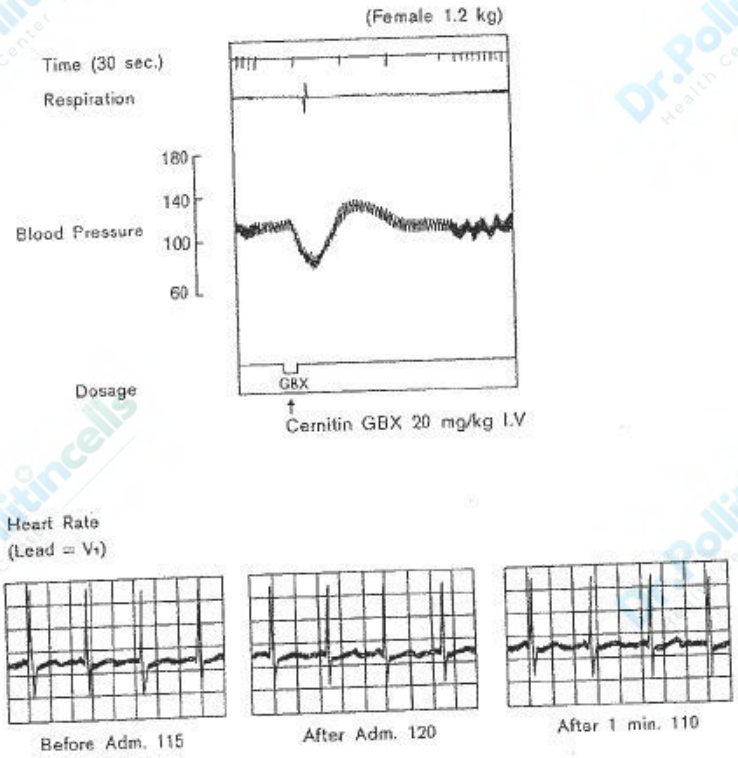


Fig. 8. Influence of Cernilton on Blood Pressure and Respiration (cat).

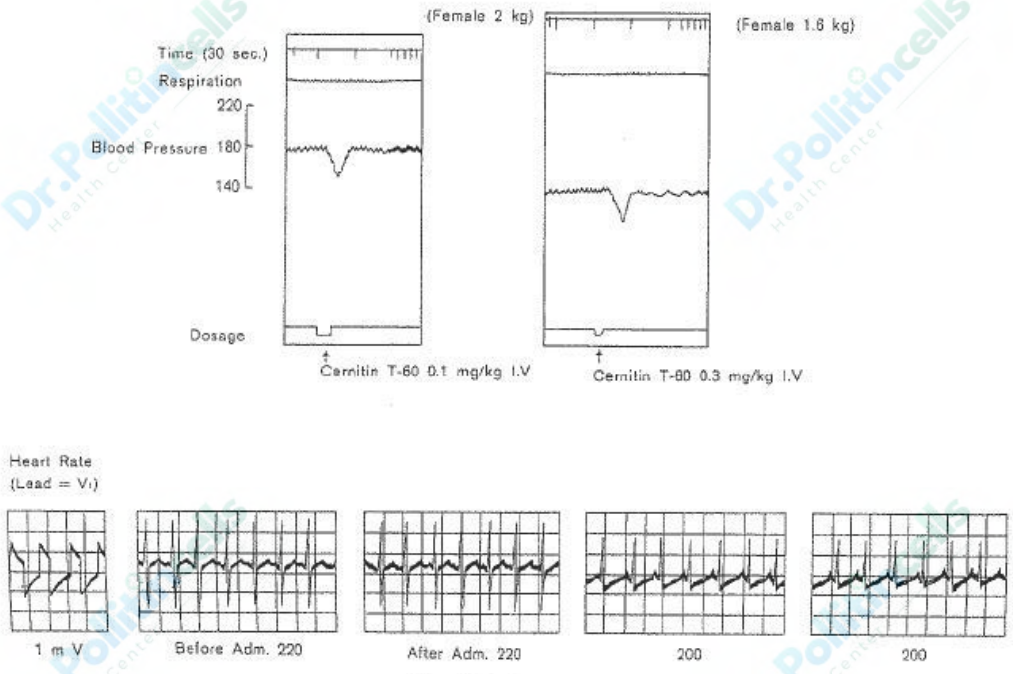


Fig. 9. Influence of Cernilton on Blood Pressure and Respiration (cat).

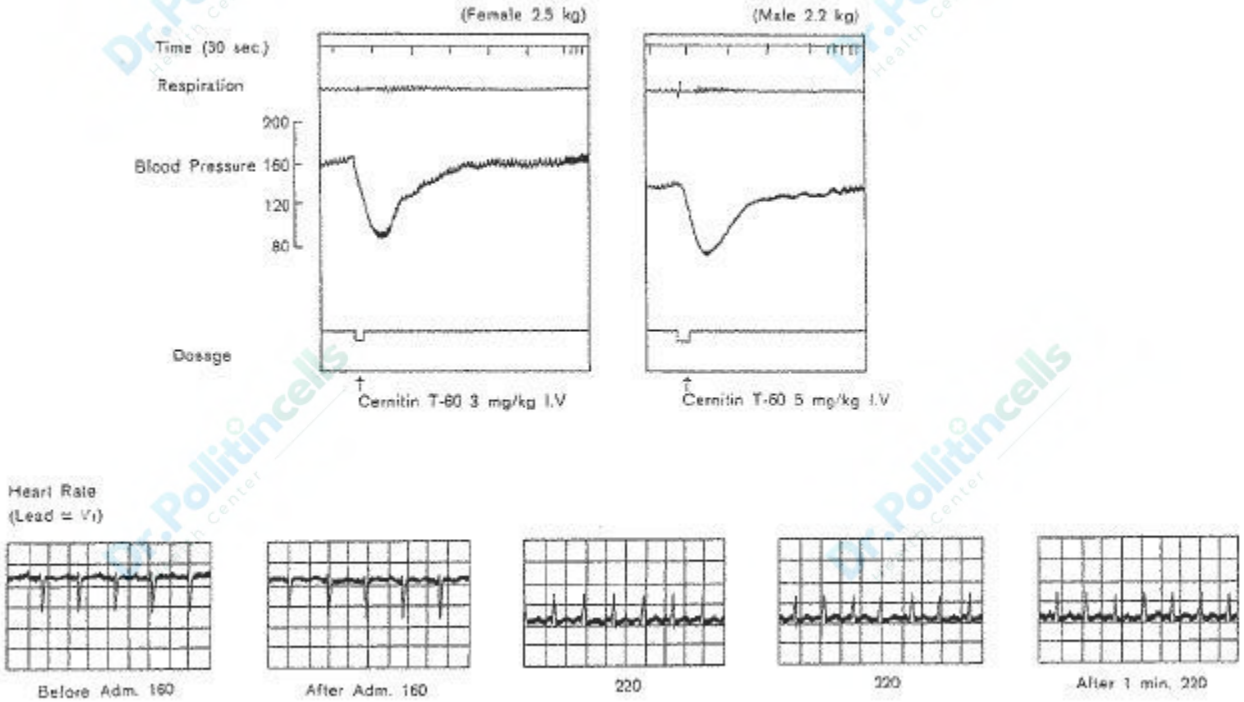


Fig. 10. Influence of Cernilton on Blood Pressure and Respiration (cat).

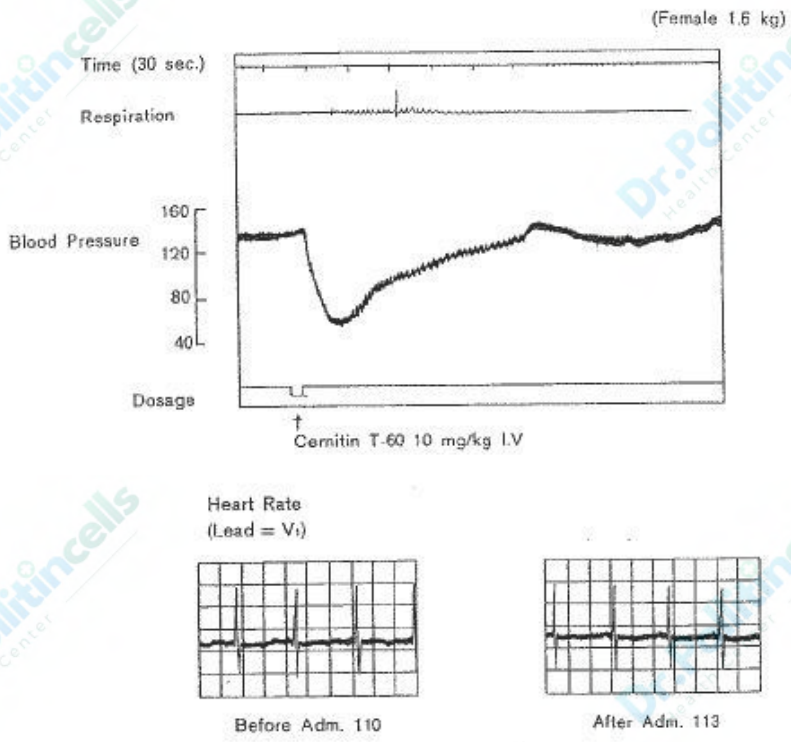


Fig. 11. Influence of Cernilton on Blood Pressure and Respiration (cat).

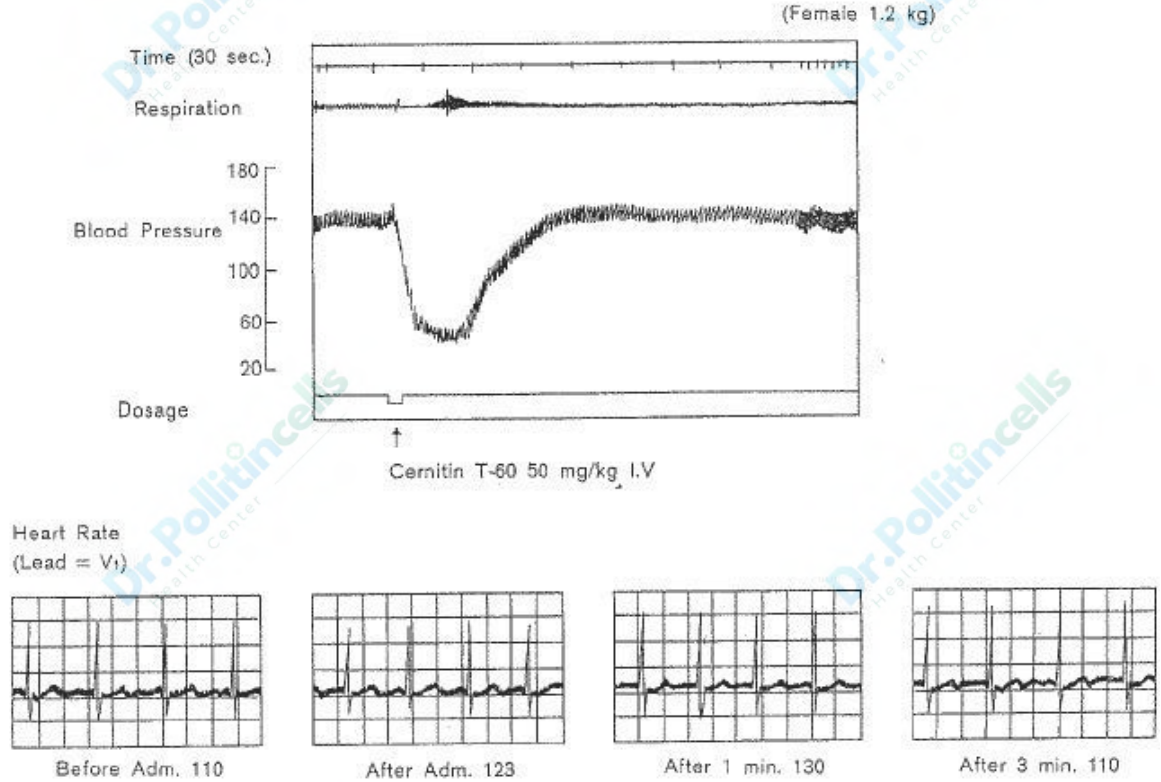


Fig. 12. Pressure Lowering Action of Cernilton

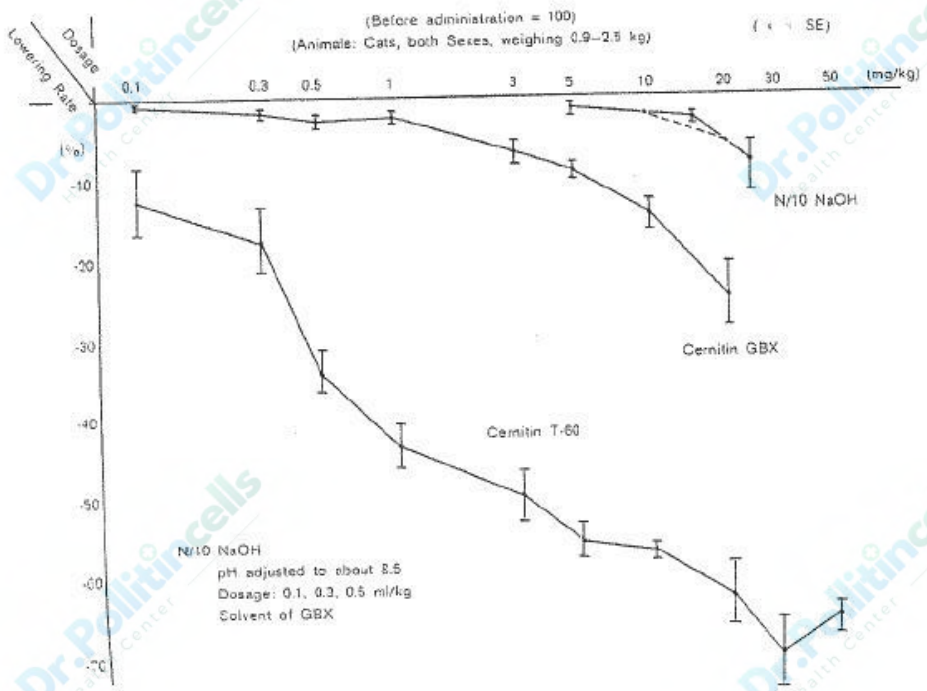


Fig. 13. Influence of T60 on Isolated Intestine (Guinea pigs)

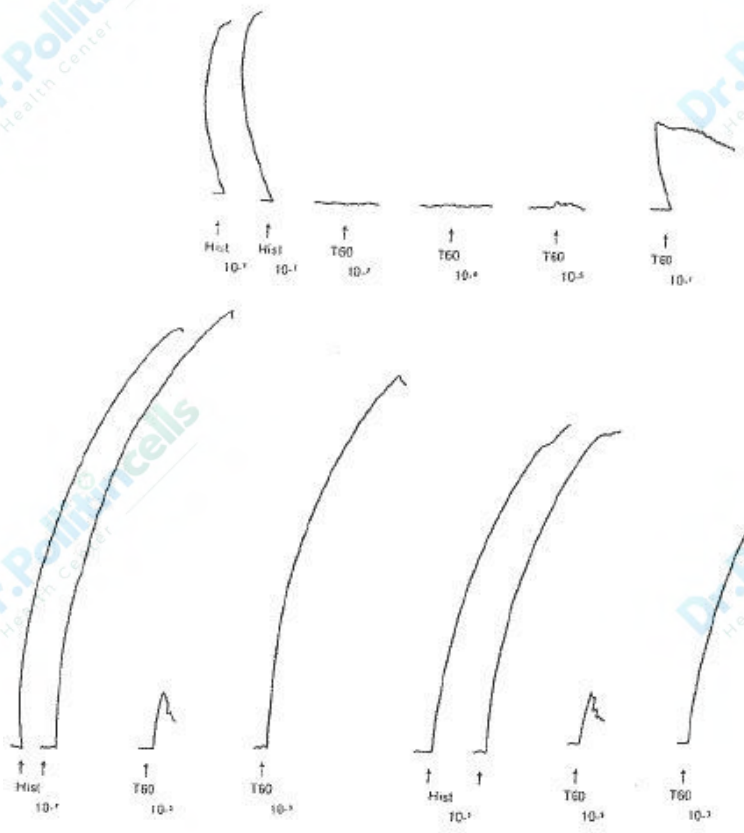


Fig. 14. Influence of T60 on Isolated Uterus (Guinea pigs)

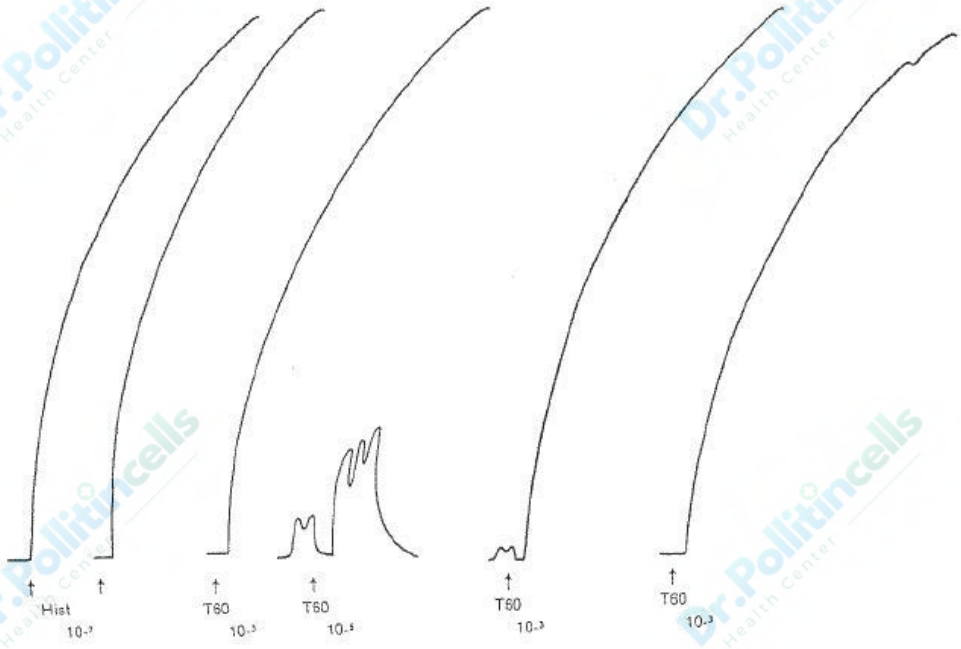


Fig. 15. Influence of T60 Isolated Bronchus (guinea pigs)

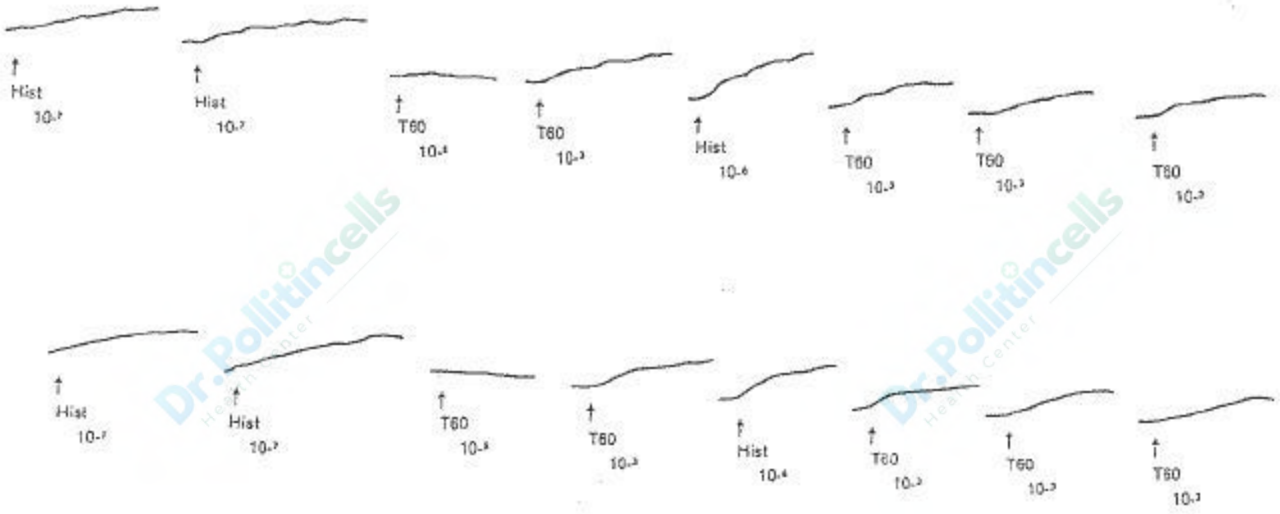
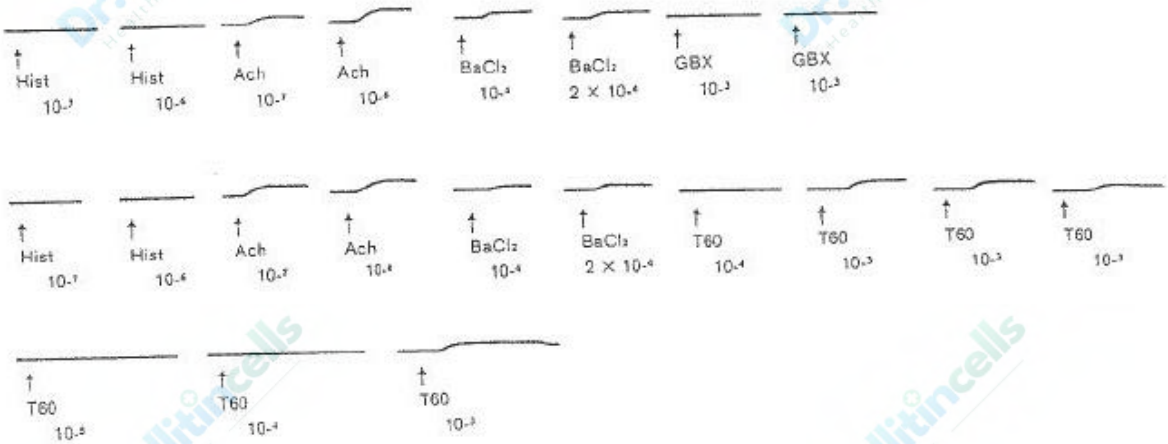


Fig. 16. Influence of GBX and T60 Isolated Prostate (rats)



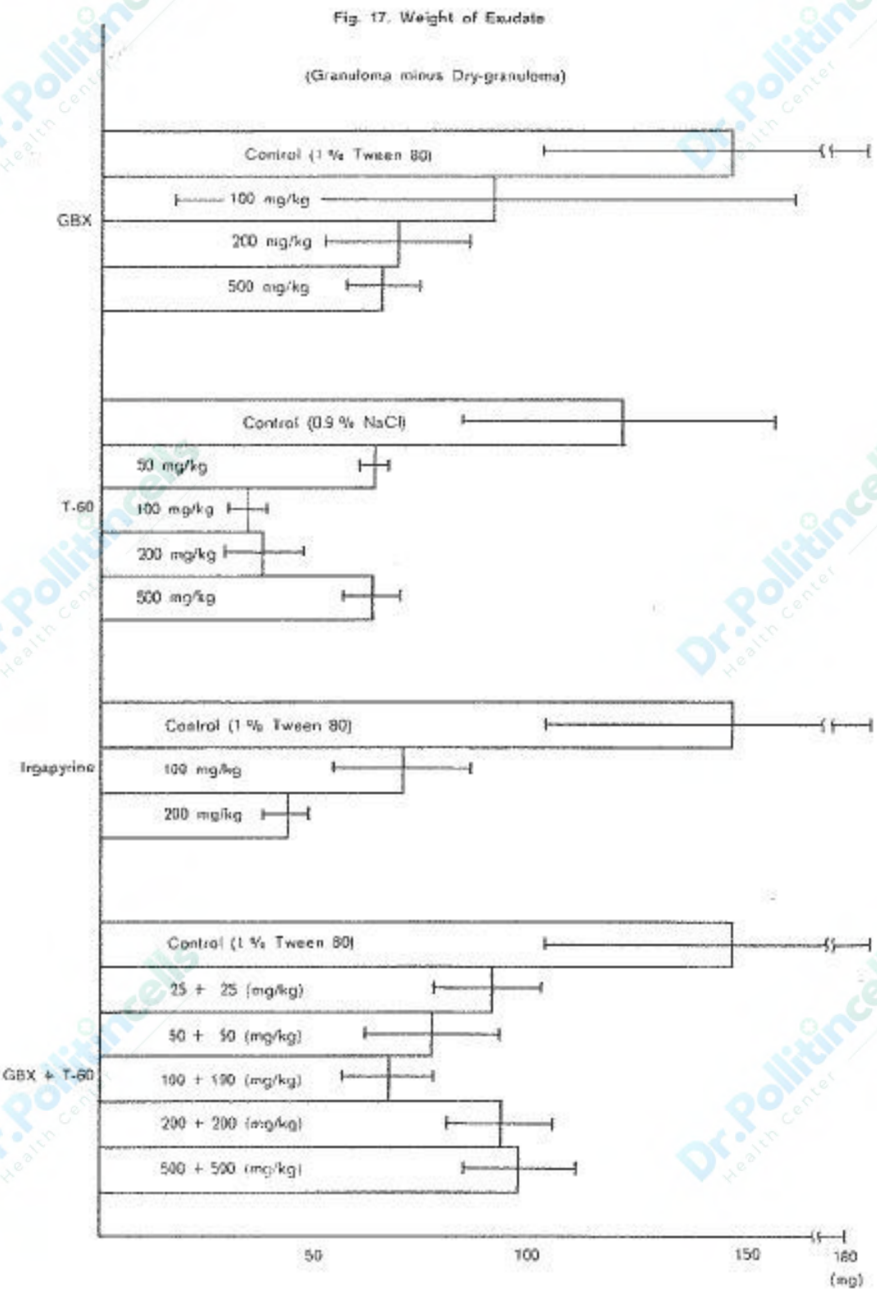


Fig. 17. Weight of Exudate
(Granuloma minus Dry-granuloma)
Control (1 % Tween 80)
100 mg/kg
GBX
200 mg/kg
500 mg/kg
Control (0.9 % NaCl)
50 mg/kg
T-60
100 mg/kg
200 mg/kg
500 mg/kg
Control (1 % Tween 80)
Ingapyrine
100 mg/kg
200 mg/kg
Control (1 % Tween 80)
25 + 25 (mg/kg)
50 + 50 (mg/kg)
GBX + T-60
100 + 100 (mg/kg)
200 + 200 (mg/kg)
500 + 500 (mg/kg)
50
100
150
180
(mg)

COLD SYMPTOM SUPPORT:

GRAMINEX Flower Pollen Extract

General immunological properties of fat-soluble (Cernitin GBX) and water-soluble (Cernitin T60) pollen extracts

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The immunological properties of fat-soluble (Cernitin GBX) and water-soluble (Cernitin T60) pollen extracts were examined *in vivo* and *in vitro*. For investigations *in vitro* we used the water-soluble pollen extract (T60), and *in vivo* the fat-soluble form (GBX). The aim of the *in vivo* experiments was to evaluate their effect on IgG antibody production, their capability of rosette formation (E-RFC) and cell indicating IgM-plaqueforming cells (PFC) hemolysins. Also observations were made on the effect of the pollen extract on graft *vs* host reaction, the transplantation barrier and delayed hypersensitivity in relation to sheep red blood cells (SRBC). Its assumed effect on phagocytosis and blastic transformation was assessed *in vitro*. In both *in vivo* and *in vitro* systems the influence of T60 and GBX preparations on the population of T- and B-cells was tested. A relationship was revealed between immunological activity and the evaluated Cernitins. Both the Cernitins examined demonstrated moderate immunoregulatory properties, but the immunosuppressive component was predominant.

Keywords: pollen extracts; immunological properties.

INTRODUCTION

Cernitins (pollen extracts obtained from A.B. Cernelle, Vegeholm, Sweden), contain water-soluble (Cernitin T60) and fat-soluble (Cernitin GBX) substances. The aim of the present work was to examine the effect of Cernitins on immunological parameters *in vivo* and *in vitro.* Previously it had been published that Cernitins given orally or intraperitoneally inhibited or counteracted the elevation of aminotransferase activity and the inflammatory process, necrosis and steatosis of liver cells (Wójcicki *et al.,* 1985).

MATERIALS AND METHODS

Effect on the formation of precipitins (IgG). Investigations were carried out on mice of the 129 Ao/Boy strain. The titre of precipitins was detected (every week) by the Ouchterlony (1949) method modified by Wilson and Pringle (1954). Animals were injected subcutaneously with a 1% solution of ovalbumin, 1 mL/kg three times a day every third day. Cernitin GBX was administered 10 mg/kg i.p. from day 3 once a day, for 21 days.

Effect on plaque forming cells (Mishell and Dutton, 1967). A plaque forming cell test was performed according to Jerne and Nordin (1963). Examinations were carried out on mice of 129 Ao/Boy strain. Cernitin GBX was administered i.p. 10 mg/kg, starting from day 1 and afterwards once a day for 4 days. On day 0 a sensitizing dose of 10% sheep erythrocytes was injected i.p. (0.2 mL per mouse). On the fourth day the test proper was carried out, using the spleen cells.

Rosette E-forming test. The experiment was carried out according to Bach and Dardenne (1972) on mice of the 129 Ao/Boy strain. Cernitin GBX was administered i.p. 10 mg/kg, starting from the day 1 and afterwards once a day for 4 days.

Determination of T- and B-lymphocytes (Boyum, 1976; Gorer and O'Gorman, 1956; Pasternak, 1969). ***In vivo*****.** Mice of 129 Ao/Boy strain received Cernitin GBX for 4 consecutive days (10 mg/kg i.p.). Lymphocytes were isolated by centrifugation (800 revs per min for 5 min at 4˚C) over a Picoll gradient. T-cells were detected by the cytotoxic test. The percentage of B-lymphocytes was determined by direct immunofluorescence. The number of fluorescent cells which were identified as B-lymphocytes is expressed in proportion to 1000 counted cells. *In vitro.* Cernitin T60 was added at 0.6, 3.0 and 15.0 mg/mL final concentration in Hanks' solution. The detailed procedure of T- and B-lymphocyte determination was described by Hoffman and Kunkel (1976).

Skin graft test (Marckman, 1966; Plużańska, 1969). Investigations were carried out on young mice of 129 Ao/Boy strain. Skin grafts of 1 cm^2 in size were transplanted in the allogenic system of mice of C_{57} B1 strain. Mice-recipients were treated with Cernitin GBX (10 mg/kg i.p.) once a day, starting 1 day before being grafted and until complete graft rejection had occurred.

Graft vs host reation (Ford *et al.,* 1970). The spleens of mice of the B_6 strain were removed and rinsed in cold HBS solution. Local graft vs. host (GvH) reaction was induced by injecting 0.05 mL of a suspension of these cells in HBS solution into the right footpad of Balb/CxB_6 mice. The right (test) and left (control) popliteal lymphnodes were removed and weighed 6 days post injection. Cernitin GBX was administered at a dose of 10 mg/kg i.p. daily for 6 days starting one day before the injection in the footpad. Results were expressed by the difference between the mean weights of the left and right popliteal nodes.

Delayed hypersensitivity test on sheep erythrocytes (SEC) (Papadimitriou *et al., 1983).* 0.2 mL of a 10% SEC solution was injected intravenously into mice of the 129 Ao/Boy strain on day 0. After 4 days, 0.05 mL of a 50% SEC solution was injected subcutaneously into a hind leg footpad: the thickness of the footpad was measured in mm after 24 h and 48 h. 10 mg/kg Cernitin GBX or 50 mg/kg Cernitin T60 were injected i.p. into the mice on day 1 and on every other day afterwards.

Blastic transformation. Experiments were carried out with human venous blood by the isotope method according to Hersh and Oppenheim (1965), modified by Plużańska (1969). Cernitin T60 was added in a Parker solution to the culture in concentrations of 0.6, 3.0 and 15.0 mg/mL 1 h before the application of 20 µg/mL phytohemagglutinin A (PHA) to the culture. The cultures were incubated for 48 h at 37˚C in a 5% CO_2 atmosphere. ^{14}C-labelled thymidine was added and incubation was continued for another 24 h under the same conditions. Radioactivity was determined by scintillation counting and expressed as counts per min.

Phagocytic activity. The investigations were carried out according to Steuden's (1978) method. The test proper was carried out using 0.1 mL of *Staphylococcus aureus* bacteria (5 x 10^8 per mL) and 0.1 mL of Cernitin T60 in concentrations of 0.6, 3.0 and 15.0 mg/mL + 0.1 mL of granulocyte solution obtained from the peritoneal exudate of a guinea-pig which had received beuillon i.p. The cultures were then centrifuged at 3000 revs per min for 30 min and 37˚C. Radioactivity was measured in 1 mL of supernatant in a Beckman scintillation counter, type 3801. Results were expressed as the percentage of phagocytosed bacteria.

Statistical analysis. Statistical analysis of the data was performed using Student's t-test. In all comparisons, $p < 0.05$ was considered to be significant.

RESULTS

The effect on the formation of precipitins. It was shown that the titre of precipitins was virtually unchanged in animals receiving Cernitin

GBX; the titre oscillated between 1:128 and 1:512, both in mice treated with Cernitin GBX and in the control group.

The effect on the plaque formed cells (PFC) and on the rosette forming cells (RFC). Cernitin GBX injected i.p. into mice affected the number of cells producing hemolysins (PFC) to some degree and also the ability of lymphocytes to form RFC with sheep erythrocytes. However, the results were contrasting: a marked increase in PFC was demonstrated while the number of RFC was reduced. The differences were statistically significant ($p<0.05$) in both cases (Table 1).

Table 1. Effective of Cernitin GBX on the plaque forming cells (PFC) and the rosette E forming cell (E-RFC)

Treatment	PFC (2×10^6 cells)	%	RFC (2×10^3 cells)	%
Cernitin GBX (10 mg/kg, $n = 10$)	771[a]	323	3.5[a]	19.4
Control ($n = 10$)	220	100	18.0	100.0

[a] $p < 0.05$.

The effect of Cernitin GBX (*in vivo*) and Cernitin T60 (*in vitro*) on the T- and B-lymphocyte subpopulations. Cernitin GBX (10 mg/kg) and Cernitin T60 (0.6-15.0 mg/mL) did not change significantly ($p>0.05$) either the number of T- and B-lymphocytes or the lymphocytes of the peripheral blood possessing no receptor (null) (Table 2).

Table 2. Influence of the Cernitin GBX (*in vivo*) and Cernitin T60 (*in vitro*) on the T- and B-lymphocyte subpopulations

Treatment	Lymphocyte subpopulations (%) T	B	Null
Cernitin GBX (10 mg/kg, $n = 10$)	64	24	13
Control ($n = 10$)	64	27	11
Cernitin T60 (mg/mL)			
0.6 ($n = 5$)	58	21	21
3.0 ($n = 5$)	63	19	18
15.0 ($n = 5$)	67	12	21
Control ($n = 5$)	60	22	18

Skin graft test and graft host reaction (GxH). In animals receiving Cernitin GBX, the rejection time of the skin graft was somewhat prolonged ($p>0.05$). The index of GvH was decreased under the influence of this preparation, but in comparison with the control group there was no statistically significant difference ($p>0.05$) (Table 3).

Table 3. Influence of Cernitin GBX on the skin graft rejection and graft vs host reaction (GvH)

Treatment	Rejection time of the skin graft (days)	GvH index
Cernitin GBX ($n = 10$)	11.0	1.2
Control ($n = 10$)	10.6	1.8

Delayed hypersensitivity to SEC test. *In vivo* Cernitin GBX and Cernitin T60 did not intensify the response to SEC. The index of the increased in footpad thickness was unchanged after 24 h, while after 48 h it was even slightly diminished ($p>0.05$) in comparison with the control group (Table 4).

Table 4. Effect of the Cernitin GBX and Cernitin T60 on delayed hypersensitivity against SEC

Treatment	Dose (mg/kg)	Index of the increase of thickness of footpad After 24 h	%	After 48 h	%
Cernitin GBX ($n = 10$)	10	4.5	100	3.0	85.7
Control ($n = 10$)	—	4.5	100	3.5	100.0
Cernitin T60 ($n = 5$)	50	4.5	100	3.0	85.7
Control ($n = 5$)	—	4.5	100	3.5	100.0

Blastic Transformation. Cernitin T60 markedly decreased blastic transformation *in vitro* expressed as the number of impulses for [^{14}C] thymidine per min. Nonspecific induction of blastic transformation with phytohemagglutinin (PHA) confirmed, to some degree, the results obtained. But the interaction between Cernitin T60 and PHA was irregular and depended on the concentration of the preparation. However, the suppressive component was predominant (Table 5).

Table 5. Effect of Cernitin T60 on the blastic transformation (isotope method)

Preparation	Concentration (mg/mL)	Counts per min
Cernitin T60 ($n = 5$)	0.6	1060[a]
Cernitin T60 ($n = 5$)	3.0	857[a]
Cernitin T60 ($n = 5$)	15.0	170
Control ($n = 5$)		227
PHA ($n = 5$)		7108
PHA + Cernitin T60 ($n = 5$)	0.6	4934
PHA + Cernitin T60 ($n = 5$)	3.0	8934
PHA + Cernitin T60 ($n = 5$)	15.0	1648[a]

[a] $p < 0.05$.

Phagocytic activity. The *in vitro* process of *Staphylococcus aureus* phagocytosis by guinea-pig granulocytes of the peritoneal exudates was completely inhibited by Cernitin T60 in concentrations of 0.6-15.0 mg/mL.

DISCUSSION

The definite anti-inflammatory effect of Cernitin extracts was demonstrated by the *Croton* oil-induced edema test (Itch, 1968). In the cotton pellet test, Cernitin T60 showed an anti-inflammatory activity in rats corresponding to the inflammation-inhibiting effect of phenylbutazone. But T60 was completely devoid of toxicity (Glømme and Rasmussen, 1965). It was also possible to confirm the anti-inflammatory effect of Cernitins on carrageenin-induced edema in rats (Dessi, 1971). Cernitins administered orally to rats demonstrated a marked antiinflammatory effect compared to the very active anti-inflammatory agents injected intraperitoneally as controls. Cernitins also inhibited the inflammatory process induced by galactosamine administration to rats (Wójcicki *et al.,* 1985). The results obtained in this experiment show that Cernitin GBX and Cernitin T60 are able to affect the course of the induced immunological processes. Such an effect is, however, defined and conditioned by the test type used and by the dose applied.

The number of RFC was affected by Cernitin GBX in a quite contrasting way. An increase in the number of PFC was accompanied by a reduction in the RFC. One may argue that the preparation examined plays an essential role in immunological processes due to regulation of the reciprocal relationship between both kinds of cells. Thus, Cernitin GBX may have a quite significant immunomodifying function.
The number of RFC was affected by Cernitin GBX in a quite contrasting way. An increase in the number of PFC was accompanied by a reduction in the RFC. One may argue that the preparation examined plays an essential role in immunological processes due to regulation of the reciprocal relationship between both kinds of cells. Thus, Cernitin GBX may have a quite significant immunomodifying function.

T- and B-lymphocytes are the morphological basis of the immunologic process. Cernitin GBX (*in vivo*) and Cernitin T60 (*in vitro*) did not alter the reciprocal relationship between the above mentioned subpopulations of lymphocytes. This could mean that another factor modulating reactivity is present or that the changes are not sufficiently marked as to be shown by the quantitative difference between the subpopulations of T- and B-lymphocytes. These results may be due to an ability to produce lymphokins, mainly interleukin 2 (Wybran and Schandene, 1985) rather than differences in morphological element

Cernitin GBX did not influence the barrier of a graft, although slight changes of graft host reaction were noted. Thus, Cernitin GBX did not change essentially either the graft reaction of the graft host reaction. The delayed reaction of hypersensitivity against SEC was not modified by the examined Cernitins. On the other hand, the blastic transformation was affected by Cernitin T60. It was reduced *in vitro* proportionally to the concentration of Cernitin T60. In relation to phytohemagglutinin a two-phase reaction was observed. Cernitin T60 applied in both low concentration and especially in high concentration, diminished the reaction, while the intermediate concentration was not effective. Thus, our observations confirm the results obtained by Kimura and Inoue (1968) demonstrating the lack of allergenic properties of both Cernitins. Our studies showed, however, that there is a relationship between the immunological system and the Cernitins tested. We can conclude, therefore, that the pollen extracts effectively possess an immunotropic/ immunoregulatory component. They show, *in vitro*, a slight immunosuppressive effect (E-RFC)—B-lymphocyte antagonism in reaction to blastogenic effect of PHA—and occasionally they act as a stimulator (PEC, blastic index). In some experimental systems they are ineffective (GvH, transplantation barrier, SEC test).

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