Science Journal Publication
Science Journal of Medicines and Clinical Trials

Article ID: 2011-2317

Research Article

The Influence of Egyptian Propolis on Induced Burn Wound Healing in Diabetic Rats; Antibacterial Mechanism

Emad T.Ahmed[1], Osama M.Abo-Salem[2], Ali Osman[1]

[1]Physical Therapy for Surgery Department, Faculty of Physical Therapy, Cairo University, Egypt.
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Al-Azhar University, Egypt.

Correspondence should be addressed to Emad T.Ahmed, emadtawfik72@yahoo.com

Accepted 12 March, 2011

Abstract

The purpose of the current study was to determine the effectiveness of Egyptian propolis in treating diabetic burn wound. Thirty eight male albino rats were participated in this study for a treatment period of 25 days. They were divided randomly and equally into 4 groups. Rats in the first group had been treated with physiological saline solution (0.9 % Na Cl) daily and served as control group. While patient in 2nd, 3rd, 4th, group received Egyptian propolis, Dermazine (silver sulfadiazine), and Propolis- Dermazine mixture creams daily. Burn surface area and bacterial colony count were used to measure the outcomes at different time interval. Results showed that a significant reduction in both burn surface area and bacterial colony count in creams treated groups compared to control group. Propolis cream was more effective than Dermazine cream, especially on D14 and after that. Interestingly, Propolis- Dermazine mixture significantly decreased bacterial colony count and burn surface area compared to propolis and Dermazine treated group. It could be concluded that propolis alone or in combination with Dermazine is considered a unique antibacterial cream for the treatment of diabetic burn wound.

Key Words: Burn - Diabetes – Wound healing – Propolis - Dermazin

Introduction

The infection of burn wounds with multiple organisms, with superadded problem of drug resistance, indicates the institution of a drug policy by the hospitals for burned patients. Gram- positive bacteria are predominant in colonization of the burn wounds. In the patients with more than 40% of total body organism[33].Wound healing is impaired in diabetic patients by different mechanisms, although recent studies have implicated a lack of Keratinocyte Growth Factor and Platelet-Derived Growth Factor function in the wound. Many of these patients have microvascular occlusive disease that may cause ischemia and impaired repair[32].Wound healing is a process that involves inflammation, proliferation/regeneration and finally remodeling. The normal orderly pattern is disrupted in chronic non-healing wounds, which are characterized by decreased proteases[4,17].Propolis, a complex resinous material collected by honeybees from buds

and exudates of certain plant. Propolis includes; fatty and phenolic acids and esters, substituted phenolic esters, flavonoids (flavones, flavanones, flavonols, dihydroflavonols, chalcones), terpenes, β-steroids, aromatic aldehydes and alcohols, and derivatives of sesquiterpenes, naphthalene and stilbenes [2,3,12, 19].

It was reported that propolis enhance immune system activities[34,23,24,25,26], oxygen radical scavenging[21,5], antimicrobial, cytostatic, anticarcinogenic, anti-inflammatory and antitumor activities [18,29,8,22,6,7,28,26].

Silver sulfadiazine (SSD) is the topical agent of choice in severe burns and is used almost universally today in preference to compounds such as silver nitrate and mafenide acetate. SSD cream, while being effective, causes some systemic complications which include neutropenia, erythema sultiforme, crystalluria and methaemoglobinemia [10,13].

Skin burn wounds appear to be the perfect model for the clinical examination and laboratory testing of healing and antimicrobial properties of drugs. Therefore, antimicrobial activity of propolis applied onto burn wounds in rats was evaluated on the basis of clinical and microbiological examinations.

The aim of the present study was to investigate the healing rates of wounds treated with three topically applied creams (propolis, dermazin, and propolis- dermazin mixture) against physiological saline solution clinically and microbiologically in diabetic rate model

Experimental design and animal groups:

Animals:

Thirty two adult male Albino Wistar rats were included in this study to determine the proper healing agent in the treatment of diabetic burned wound. All rats were admitted to the study approximately 6 month of age (considered adult). Free access was allowed to standard diet, water, temperature, humidity and light period. The rat model was considered appropriate for simulating wound healing in humans. The animal model was selected for this study to provide better control over confounding variables such as diet, exercise, environmental stress and systemic disease except diabetes which affect wound healing. Four groups were used (8 animals/group). The control group received physiological saline solution and the three treatment groups received propolis, dermazine, propolis- dermazine mixture creams one time/day/25 days.

Induction of diabetes:

Type 1 diabetes mellitus was induced by intraperitoneal injection of streptozotocin (60 mg/kg body weight) in citrate buffer 0.1 mol/ L (pH 4.2) for 3 successive days1. Rats were considered diabetic if blood glucose concentrations increased to 200 or more mg/ dl.

Induction of burn:

Each animal was anesthetized by placing the animal into closed cage in association with a piece of cotton wetted ether. The animals were strictly tied via four cotton threads to a wooden plate. The hair at the upper part of left hind limb was removed by hair removal cream (veet), manufactured by EVA cosmetic EGYPT and the skin were cleaned by a piece of cotton wetted with alcohol. The area of the skin prepared as above equal 4 cm2 while the area intended to be burned equal 2 cm2 , however, this will ease the procedure of burning as well as measurement. The burns were induced by a rectangular metal seal with cm2 contact surface area that was heated on a flame burner to 45 ◦ C and handed by a surgical forceps, then pressed immediately against the prepared skin segment for 20 seconds to produce partial thickness burns according to Kloth and Feeder16 protocols. Each animal caged separately and they were subjected to a normal day light rhythm, and the room temperature varied between 32◦ C to 35 ◦ C.

Materials and Methods

Topical treatment cream:

Egypt propolis used in this study was obtained from faculty of agriculture Cairo Egypt. Extracts of propolis sample was prepared and used throughout this work as described by ISLA et al15 with minor modifications. Propolis was frozen at -20°C, and ground in a chilled mortar. Then, the round powder was extracted with ethanol (15 ml of 80 % ethanol/ g of propolis) with continuous stirring at room temperature for 24 h. The suspension was let to sediment for 3 days. The supernatant was then concentrated in a evaporator under reduced pressure at 40°C and the residue was mixed within same amount of 50 % cold cream (Botafarma, cold creme, 12.5 % spermaceti + 12 % white wax + 56 % liquid paraffin + 0.5 % borate of soda + 19 % distilled water).

2- Dermazin cream: 1 % Silver Sulphadiazine, manufactured by MUP company , EGYPT.

3- Propolis – Dermazin mixture cream : 50 % of propolis cream was mixed with 50 % Dermazin cream.

4- Physiological saline solution: Normal saline (NS) is the commonly-used term for a solution of 0.9% of Na Cl

Procedure of the study:

Immediately after burn, the burned areas in the first group (control group) received physiological saline solution daily, the 2nd, 3rd, and 4th group received propolis, Dermazine, and propolis-Dermazine mixture cream respectively.

Evaluation:

Burn surface area (BSA): The measurement of BSA was conducted by tracing of wound perimeter according Kloth and Feedar16. The BSA measurement was conducted by the following steps:

A sterilized transparency film was placed over the ulcer. The ulcer perimeter was traced by using the film tipped transparency marker. Each ulcer was traced three times to establish measurement reliability. After tracing the transparency film face which faced the ulcer was cleaned by a piece of cotton and alcohol. The carbon paper was placed over the metric graph paper one mm2. The traced transparency film was placed over the carbon paper with a white paper in between, and transcribed the tracing onto the metric graph paper. The number of square millimeters on the metric graph within the wound tracing was counted to determine the BSA. The mean of the three trials was calculated and considered as BSA. The BSA measurements were taken at day 4, 8, 14, 19 and 24 days post burn.

Wound infection assessment:

Quantitative bacteria culturing of wounds was performed at different time interval, so as not to disturb the wound environment, on day 5, 10, 15, 20 and 25. Swabs specimens for bacterial cultures were collected before cleaning of wounds by the use of sterile sticks, which were rolled over the wound each time it is aseptically placed in a tube containing 5 mL of saline. Using a 100-fold dilution, finally 0.1 mL were pipetted and inoculated on nutrient Agar plate and grown aerobically at 37°C for 24 h. Bacterial colonies were counted at x105 bacterial organism/ mL (Paul and Gordon, 1978).

Data analysis:

This study was a controlled post test experimental design with a control and a three treatment groups. Groups were compared for differences at different time interval, ANOVA multiple comparisons followed by Tucky Kramer post hoc test was used for comparing differences between 3 treatment groups and control group. The level of significance was set at 0.05 for all statistical tests.

Results:

Type 1 diabetes was induced as described in the material and methods. All rats in the experiment were selected with a closure blood glucose level and it was no significant difference between groups using ANOVA test.

Normal rat	Control (PSS)	PC	DC	DC & PC
64.13 ± 4.04	220.63 ± 6.08	222.63 ± 6.82	223.88 ± 12.40	221.25 ± 6.66

Table (1): Serum blood glucose level

Data were expressed as Means ± SEM of 8 diabetic rats /group. C; PSS treated group (control group), PC; propolis cream treated group, DC; dermazin cream treated group, PC & DC; propolis-dermazin mixture cream treated group. Significance was carried out by One-way ANOVA Tukey-Krammer test.

Burn was induced as described in the material and methods in diabetic rats and treated with a topical cream daily. Rats were divided into four group, each group treated with a different cream for study periods (25 days), sampling for bacterial culture was taken at specific day intervals as explained in the table. Data in table (2) showed that all creams used significantly reduced bacterial colony count as compared to physiological normal saline (PSS) treated control group. There was a significantly difference between treated groups as shown in table (2).

Groups treated with a mixture of DC & PC were significantly reduced bacterial count at all days of evaluation compared to PSS (physiological saline solution), PC and DC groups. While, PC and DC groups were significantly reduced the bacterial colony count on day 15, 20 and 25 compared to control group. . Interestingly; BCC in PC treated group was significantly lower than BCC in group treated with DC at time D15, D20 and D25.

The duration of the treatment was significantly effective in the reducing the bacterial colony count in cream treated groups, but increased in the PSS treated group (table 2). Bacterial colony count were significantly reduced on days 15, 20 and 25 of the experiment for groups treated with PC and DC and days 10, 15, 20 and 25 for group treated with a mixture PC & DC compared to the PSS treated group. Moreover, it was a significant difference in BCC at different time intervals between different cream treated groups.

Table (2): Bacterial colony count (10^5) from experimental burns using diabetic rats, at each bandage change, following treatment (every day) with various topical creams.

Days (D) of sampling	Topical treatment			
	Control (PSS)	PC	DC	PC & DC
D5	229.38± 5.58	210.88± 7.61	214.25± 8.52	177.75 ± 9.15^{a,b,c}
D10	230.88± 8.33	212.38 ± 7.35	228.00 ± 10.85	109.50 ± 9.40$^{a,b,c;\dagger}$
D15	311.75 ± 9.43†,‡	91.13± 6.30$^{a;\dagger,\ddagger}$	162.88± 14.69$^{a,b;\dagger,\ddagger}$	52.63± 5.24$^{a,b,c;\dagger,\ddagger}$
D20	338.25 ± 10.87†,‡	58.38 ± 4.94$^{a;\dagger,\ddagger,\neq}$	145.25 ± 6.85$^{a,b;\dagger,\ddagger}$	15.38± 1.96$^{a,b,c;\dagger,\ddagger,\neq}$
D25	412.38 ± 12.72$^{\dagger,\ddagger,\neq,\#}$	20.50 ± 1.98$^{a;\dagger,\ddagger,\neq,\#}$	71.50 ± 6.36$^{a,b;\dagger,\ddagger,\neq,\#}$	10.00 ± 1.22$^{a,c;\dagger,\ddagger,\neq}$

Data were expressed as Means ± SEM of 8 diabetic rats /group. C; PSS treated group(control group), PC; propolis cream treated group, DC; dermazin cream treated group, PC & DC; propolis-dermazin mixture cream treated group.

significantly different versus control group; significantly different versus PC group; significantly different versus DC group at $P \leq 0.05$.

[†]significantly different versus D5; [‡]significantly different versus D10; [≠]significantly different versus D15; [#]significantly different versus D20 at $P \leq 0.05$. Significance was carried out by One-way ANOVA Tukey-Krammer test.

Burn surface area (BSA) was measured at specific day intervals as explained in the table. Data in table (3) showed that all creams used significantly reduced BSA as compared to physiological normal saline (PSS) treated control group except on D4. PC & DC a mixture cream significantly reduced BSA at time D14, D19 and D 24 compared to PC and DC treated group except PC at D14. Interestingly; BSA in PC treated group was significantly lower than BSA in group treated with DC at time D19 and D24.

The duration of the treatment was significantly effective in the reducing BSA in cream treated groups, but increased in PSS treated group (table 3). PC and a mixture of PC & DC were significantly reduced BSA at D14 compared to D4 and D8, and it was also

Table (3): Burn areas from experimental diabetic rats, at each bandage change, following treatment (every day) with various topical cream

Days (D) of measurements	Topical treatment cream Burn surface areas (BSA) (cm^2)			
	Control (PSS)	**PC**	**DC**	**PC & DC**
D4	**2.21 ± 0.08**	**2.03 ± 0.07**	**2.14 ± 0.08**	**2.01 ± 0.07**
D8	**2.52 ± 0.09**	**1.98 ± 0.10**[a]	**2.04 ± 0.09**[a]	**1.79 ± 0.09**[a]
D14	**3.04 ± 0.14**[†,‡]	**1.69 ± 0.08**[a;†]	**1.95 ± 0.08**[a]	**1.40 ± 0.08**[a,c;†,‡]
D19	**4.04 ± 0.13**[†,‡,≠]	**1.29 ± 0.05**[a;†,‡,≠]	**1.71 ± 0.14**[a,b]	**0.89 ± 0.03**[a,b,c;†,‡,≠]
D24	**4.13 ± 0.10**[†,‡,≠]	**0.86 ± 0.07**[a;†,‡,≠,#]	**1.34 ± 0.14**[a,b;†,‡,≠]	**0.54 ± 0.04**[a,b,c;†,‡,≠,#]

Data were expressed as Means ± SEM of 8 diabetic rats /group. C; PSS treated group (control group), PC; propolis cream treated group, DC; dermazin cream treated group, PC & DC; propolis-dermazin mixture cream treated group.

significantly different versus control group; significantly different versus PC group; significantly different versus DC group at $P \leq 0.05$.

[†]significantly different versus D5; [‡]significantly different versus D10; [≠]significantly different versus D15; [#]significantly different versus D19 at $P \leq 0.05$. Significance was carried out by One-way ANOVA Tukey-Krammer test.

Discussion:

Burns and post-burn wounds are the most common skin injuries. They have been the incentive for many clinical and research centers to seek a substance which would have the basic therapeutic functions, regenerative, reparative, anti-¬micro-organic and an anaesthetic, necessary for dermatolo¬gical medicaments.One of the pharmacopeial substances that have the above-mentioned properties is propolis.Apitherapeutics are used effectively in many fields of medicine. There have been reports on their usage in:

Dermatology, in purulent skin inflammations, bed sores, dermatomycosis; Gynecology, in erosion and vaginitis. Apitherapeutics are also used in pediatrics, surge¬ry, dentistry and endocrinology9.The therapeutic effectiveness of apitherapies, in which the pharmacological activity results from their physicochemical properties, was confirmed by Molan20 in his research.

The plethora of therapeutic modalities used in the treatment of burn wound especially when complicated by diabetes is in itself, testimony to lake of efficacy of any one measure. This study set out, therefore, to assess and compare the effect of propolis alone or in combination with Dermazine on diabetic burn wound healing.

The 4 groups were evenly balanced according to age, burn surface area, standard surgical care, and diabetes level. The time from the moment the thermal wound was made to the beginning of repair processes in the wound was different depending on the applied remedy. Generally, all the wounds were enlarged during the first three days of treatment, which was due to wound retraction as well as splinting of wound edges by the bandaging, but the enlargement stop to increase in the propolis, Dermazine, and propolis-Dermazine mixture cream treated group after that and continue to increase only in control (PSS) treated group. There was also a significant reduction of BSA in propolis treated group when compared to Dermazine treated group. Moreover there was a significant reduction in BSA in Propolis-Dermazine mixture cream treated group when compared to either remedy alone especially at day 24.

The above BSA finding was supported by the bacterial colony count result which stated that at day 20 and day 25, it was found that either propolis or Dermazine is effective in reducing the bacterial colony count compared to control group. Interestingly, it was found that the complementary of both agents (propolis-Dermazine mixture cream) lead to superior results in comparison with strict adherence to either agent alone.

It was concluded from the above result that propolis have antibacterial effect more than the traditional antibacterial cream (Dermazine) also, the unique combination of both Propolis and Dermazine lead to superior reduction in both BSA and bacterial colony counts especially during the proliferative phase of wound healing.

The findings of our study are consistent with several investigators. Han et al14 concluded that the application of propolis skin cream could be considered an alternative to the use of traditional antibiotic silver sulfadiazine (SSD). Takasi et al.30 stated that propolis inhibits bacterial growth by preventing cell division, thus resulting in the formation of pseudo-multicellular streptococci. In addition, propolis disorganized the cytoplasm, the cytoplasmic membrane and the cell wall, caused a partial bacteriolysis and inhibited protein synthesis.

Grange and Davey11 found that propolis have antibacterial activity against a range of commonly encountered cocci and Gram-positive rods, including the human tubercle bacillus, but only limited activity against Gram-negative bacilli. These findings confirm that, the antimicrobial properties of propolis possibly attributed to its high flavonoid content.

In summary, the emerging method of using both Propolis and Dermazine in combination cream have offered a renewed hope to patients with impaired wound healing

References

[1] Abdel Wahab, Y.H., O'Harte, F.P., Ratcliff, H., McClenaghan, N.H., Barnett, C.R., Flatt, P.R (1996). *Glycation of insulin in the islets of Langerhans of normal and diabetic animals*. Diabetes 45; 1489-1496.

[2] Aga H, Shibuya T, Sugimoto T, Kurimoto M, Nakajima SH. *(1994) Isolation and identification of antimicrobial compounds in Brazilian propolis*. Biosci Biotechnol Biochem, 58:945–6.

[3] Bankova V, Christov R, Kujumgiev A, Marcucci MC, Popov S. (1995). *Chemical composition and antibacterial activity of Brazilian propolis*. Z Naturforsch [C] 50:167–172.

[4] Bloomgarden ZT. (2004). *Diabetes complications. Diabetes Care, 27: 1506–1514*.

[5] Chen C. N., Weng M. S., Wu C.L., Lin J. K. (2004). *Comparison of the radical scavenging activity, cytotoxic effects, and apoptosis induction in human melanoma cells by Taiwanese propolis*, eCAM, (Evidence based Contemporary Alternative Medicine), 1, 175-185,.

[6] Dimov V., Ivanovska N., Bankova V., and Popov S (1992). *Immunomodulatory action of propolis: IV. Prophylactic activity against gram-negative infections and adjuvant effect of the water-soluble derivative.*Vaccine, 10, 817-823.

[7] Dimov V., Ivanovska N., Manolova N., Bankova V., Nikolov N., and Popov S., (1991). *Immunomodulatory action of propolis. Influence on anti-infectious protection and macrophage function Apidologie*, 22, 155-162.

[8] Duarte S., Rosalen P. L., Hayacibara M. F., Cury J. A., Bowen W. H., Marquis R. E., Rehder V. L., Sartoratto A., Ikegaki M., Koo H., (2006). *The influence of a novel propolis on mutans streptococci biofilms and caries development in rats Arch.Oral*. Biol., 51, 15-22.

[9] Gafar M., Guti L., Dumitri H. (1976). *Pharmaceutical preparations with propolis extracts used in treatment of peripheric chronic paradontopathies*. II In- ternational Symphosium Apitherapy. Bukarest,.

[10] Gracia C.G. (2001). *An open study comparing topical silver sulfadiazine and topical silver sulfadiazine-cerium nitrate in the treatment of moderate and severe burns.* Burns, 27, 67-74..

[11] Grange, J. M. and Davey, R. W (1990) . *Antibacterial properties of propolis (bee glue)* Journal of the Royal Society of Medicine 83, 159-160,

[12] Greenaway W, May J, Scaysbrook T, Whatley FR. (1991). *Identification by gas chromatography-mass spectrometry of 150 compounds in propolis*. Z Naturforsch [C] 46:111–121.

[13] Gregory R.S., Piccolo N., Piccolo M.T., Piccolo M.S., Heggers J.P. (2002). *Comparison of Propolis Skin Cream to Silver Sulfadiazine : A Naturopathic Alternative to Antibiotics in Treatment of Minor Burns.* J. Altern. Complem. Med., 8: 77-83.

[14] Han MC, Durmus AS, Karabulut, and Yaman I. (2005). *Effect of Turkish propolis and sulfadiazine on burn wound healing in rats*. Revue. Med. Vet. 156: 12, 624-627.

[15] Isla M.I., Moreno M.I.N., Sampietro A.R., Vattuone M.A. (2001). *Antioxidant activity of Argentine propolis extracts.* J. Ethnopharmacol. 76: 165-170.

[16] Kloth LC, and Feedar JA (1988). *"Acceleration of Wound Healing with High Voltage Monophasic Pulsed Current"* Phys Ther. 68;503-508.

[17] Lotfy M, Badra G, Burham W, and Alenz FQ (2006). *Combined use of honey, bee proplis and myrrh in healing a deep, infected wound in a patient with diabetes mellitus*. Bri. J. Biomedical science, 171-173.

[18] Marcucci M. C. (1995): Propolis, *chemical composition, biological properties and therapeutic activity* Apidologie, 26, 83-99,.

[19] Marcucci MC, De Camargo FA, Lopes CMA (1996). *Identification of amino acids in Brazilian propolis.* Z Naturforsch [C] 51:11-14,.

[20] Molan P.C(2001). *Potential of honey in the treatment of wound and burns.* Am. J. Clin. Dermatol. 2: 13-19.

[21] Moreno M., Isla M., Sampietro A., Vattuone M., (2000) .Comparison of the free radical-scavenging activity of propolis from several regions of Argentina J. Ethnopharmacol. 71, 109-114.

[22] Orsi RO, Sforcin JM, Funari SR, Bankova V. (2005). *Effects of Brazilian and Bulgarian propolis on bactericidal activity of macrophages against Salmonella typhimurium*. Internat Immunopharmacol;5:359-368.

[23] Orsolic N., Basic I. (2006). *Cancer chemoprevention by propolis and its polyphenolic compounds in experimental animals Recent Progress in Medicinal Plants, 17, 55-113,*

[24] Orsolic N., Basic I. (2003),: *Immunomodulation by water-soluble derivative of propolis (WSDP) a*

factor of antitumor reactivity J. Ethnopharmacol., 84, 265-273.

[25] Orsolic N., Kosalec I., Basic I. (2005). *Synergystic Antitumor Effect of Polyphenolic Components of Water Soluble Derivative of Propolis against Ehrlich Ascites Tumour, Biol.* Pharm. Bull., 28, 694-700.

[26] Orsolic N., Sver L., Terzic S., Basic I., (2005). *Ethanolic Extract of Propolis (EEP) Enhances the Apoptosis-Inducing Potential of TRAIL in Cancer Cells Vet. Res. Commun., 29, 575-593*.

[27] Paul, J.W. and M.A. Gordon, (1978). *Efficacy of chlorxidine surgical scrub compared to that of Hexachlorophene and Povidone-iodine*. VMC/SAC., 573: 345-356.

[28] Russoa, A., Cardileb, V., Sanchezc, F., Troncosoc, N., Vanellaa, A., and Garbarinod, J. A.,(2004). *Chilean propolis: antioxidant activity and antiproliferative action in human tumor cell lines*. LifeSci., 76, 545-558.

[29] Silici S., Kutluca S., (2005). *Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region*. J. Ethnopharmacol., 99, 69-73.

[30] Takasi, Kikuni NB; Schilr H. (1994). *Electron microscopic and microcalorimetric investigations of the possible mechanism of the antibacterial action of propolis*. Povenance planta Med., 60(3): 222-227.

[31] Wander P. (1995). *Taking the sting out of dentistry. Dental Practice, 25:3.*

[32] Werner S, Breeden M, Hubner G, Greenhalgh DG, Longaker MT (1994). *Induction of keratinocyte growth factor expression is reduced and delayed during wound healing in the genetically diabetic mouse*. J Invest Dermatol. 103:469–473.

[33] Winkler M., Erbs G., Muller F.E., Konig W. (1987).*Epidemiologic studies of the microbial colonization of severely burned patients*. Zentralbl. Bakteriol. Mikrobiol. Hyg. 184, 304 –320.

[34] Wleklik M., Zahorska R., Luczak M., (1997). *Interferon-inducing activity of flavonoids Acta Microbiol*. Pol., 32, 1141 -1148