A COMPARATIVE EVALUATION OF ANALGESIC AND ANTI-INFLAMMATORY ACTIVITIES OF RHIZOPHORA MUCRONATA BARK EXTRACTS

Rohini R M and Amit Kumar Das

Affiliation**:** Department of Pharmaceutical Chemistry, Krupanidhi College of Pharmacy, Bangalore, Karnataka, India;

Corresponding author: rm_rohini@rediffmail.com

Summary

Rhizophora *mucronata is* used for the treatment of inflammation diarrhea, and angina disorders by people of India and Burma. The analgesic and anti-inflammatory activities of the plant have not been scientifically evaluated so far. The objective of this study was to comparatively evaluate scientifically the analgesic and anti-inflammatory activities of the successive chloroform, ethyl acetate, methanol and water solvent extracts of Rhizophora *mucronata.* Analgesic activity was evaluated by Eddy's hot plate and writhing method in albino mice; The anti-inflammatory activity was evaluated by formalin induced paw edema, sponge pellet granuloma in rats, Freud's adjuvant induced inflammation in rats .The extracts were found to be nontoxic in acute toxicity study. A dose dependent 250 and 500 mg/kg p.o of methanol extract administered to the rats resulted, significant ($p<0.05$) inhibition of formalin induced rat paw edema and sponge pellet granuloma and at 500 mg/kg in Freud's adjuvant model. All the solvent extracts failed to produce significant analgesic activity. These observations prove scientifically the anti-inflammatory property of Rhizophora *mucronata* and thus provide scientific support for its traditional use.

Key words: Rhizophora *mucronata,* anti-inflammatory activity, analgesic activity

Introduction

Rhizophora mucronata commonly known as mangrove belongs to Rhizophoreace family is grown in the tropical subtropical region coastlines; and is documented for its folk remedies to treat angina, diarrhea, haemorrhage and inflammation in countries like India , Burma (1). Bark of this plant is used as a source of tannins and dyes (2) Chemical constituent like sesquiterpenoid and triterpenoids and steroids has been investigated from this plant (3). The polysaccharide of bark extract has been reported for anti HIV activity (4). However the analgesic and anti-inflammatory activity associated with bark as traditional used, has not been investigated. This study presents a comparative scientific evaluation of analgesic and anti inflammatory activity from the successive solvent extracts of the Rhizophora mucronata bark

Materials and methods

Plant material

The bark of Rhizophora mucronata was collected from the Mangalore coast of south west India. The plant was identified from the forest department of Kundapura, Managalore and was authenticated from the Department of Botany Ganynabharthi, Bangalore University, Karnataka (India).

Extraction

One kg of powdered R mucronata (RM) bark was loaded for extraction in a soxhlet apparatus using solvents from chloroform followed by ethyl acetate, and methanol and lastly boiling the dry marc with water to get the respective RMC, RME, RMM, and RMW extracts. After each successive solvent extraction the marc was dried before subjecting to extraction with the next solvent; the extracts were concentrated under vacuum and yield were noted. The extracts were refrigerated at 4 ^{0}C prior to use.

Experimental animals

Swiss mice (20 – 25 g) and Sprague dawley rats (220 – 250 g) of either sex were procured from the Pharmacology animal house, Department of Pharmacology, Krupanidhi college of Pharmacy, Bangalore, India. The animals were acclimatized to standard environmental condition; the animals had free access to standard pellet diet and water *ad libitum*. The experimental protocol was approved by ethical committee of the institution.

Acute toxicity studies

An acute toxicity study relating to the determination of LD_{50} was performed in mice following the OPPTS guidelines (5)

Preparation of the samples for the bioassay

The chloroform (RMC) and ethyl acetate (RME), methanol (RMM) and water (RMW) extracts of Rhizophora mucronata bark were suspended in 5 % Tween 80. Positive controls Tremadozole and Ibuprofen were prepared in 5 % Tween 80.

Analgesic and anti inflammatory studies

Eddy's Hot plate method (6)

Albino mice of either sex weighing 22-25 g were divided into ten groups, each group consisting of six animals. Group I served as control received 3 ml/kg suspension of 5% tween 80 in water; while group II were administered Tremadazole 5 mg/kg as positive control. The latency for stimulus was recorded before the start of the experiment and after an interval of every 30 min for duration of 1 h following oral administration of 250 mg/kg and 500 mg/kg of RMC, RME, RMM and RMW extracts of R *mucronata* bark respectively and the positive control.

Writhing tests

The method of Haire SW et al. was adopted (7). The albino mice of either sex weighing 22-25 g were divided into 10 groups (n= 6); a dose of 250 mg/kg and 500mg/kg body weights was selected for each extract of R mucronata bark. Group I served as control received a suspension of 3 ml/kg of (5% v/v tween 80 in water); group II was administered with aspirin 3 mg/kg; group III-X animals received 250 mg/kg & 500 mg/kg of RMC, RME, RMM and RMW extracts of R *mucronata* bark respectively. Prior to the treatment, acetic acid 0.1ml of 0.6 % was injected intra peritoneal as pain stimulus to all the animals. The numbers of writhes were counted for 15 min.

Paw edema method

Anti-inflammatory activity was assessed by the method described by Brownlee G (8) Male or female Sprague dawley rats with a body weight between 100-150 g were selected and were divided into ten groups (n = 6). The animals were starved overnight and water was given *ad labium.* The rats in group I were challenged with control tween 80 (5% v/v) suspension 3 ml/kg and group II received diclofenac 10 mg/kg orally while group III-X received 250 mg/kg & 500 mg/kg of RMC, RME, RMM and RMA extracts orally. The volume of the hind paw was measured using plethysmograph before injecting formalin (0.1 ml of 2% v/v in normal saline) by a subcutaneous injection into the plantar side of the left hind paw. Extracts were given orally one hour before injecting formalin. The paw volume was measured at every 1h interval for duration of 5 h interval and at 24^{th} h after challenge of the extracts and drug; fixed volume of distilled water was given to ensure uniform hydration. The difference between the initial and subsequent reading gave the actual edema volume.

Sponge pellet implantation -Induced Granuloma (9, 10)

The rats were divided into ten groups (n=6). After shaving the fur, the rats were anaesthetized and standard size and weight (20.0 ± 0.02 mg) disc shape sterile sponges were inserted, one in each axilla. The RMC, RME, RMM and RMW (200 and 500

mg/kg, *p.o.*) and ibuprofen (10 mg/kg, *p.o.*) and control vehicle were administered orally for 7 consecutive days from the day of sponge pellet implantation. The animals were anaesthetized on the eighth day and sponge pellets were removed surgically and made free from extraneous tissues. The pellets were incubated at 37 °C for 24 h and dried at 60 °C to constant weight. Increment in the dry weight of the pellets was taken as measure of granuloma formation.

Adjuvant induced arthritis – immunological method (11)

The albino rats weighing between 200-220 g were selected and divided into ten groups of six animals each. Group I served as control received tween 80 (5% v/v) in water; group II was treated with diclofenac (10 mg/kg p.o), group III-X received orally a low dose of 250 mg/kg and a higher dose of 500 mg/kg of chloroform, ethyl acetate, methanol and aqueous extracts of Rhizophora mucronata bark. All the animals were injected with 0.5 ml of Freud's adjuvant, into the sub- planter surface of right hind paw. The extracts, standard drug and control were administered orally once a day commenced on the day of injection of adjuvant and continued up to 28th post adjuvant challenge day.

The assessment of the change in the inflammatory reaction was made by measuring the paw volume plethsmographically on first, seventh, 14th and 28th day after Freund's adjuvant. A change in the inflammatory reaction was viewed by radiography (X-ray) of the injected part using a dental x-ray unit on 28th day.

Statistical analysis

The data was analyzed statistically using one-way analysis of variance followed by Dunnett's't' test. The data are expressed as mean ± S.E.M. P-values less than 0.05 imply significance.

Results

Acute toxicity study: The lower dose at 2000mg/kg body weight and higher dose of 5000 mg/kg bodyweight of each solvent extract subjected to the albino mice were found to be nontoxic.

Writhing method

The effects of RM bark extracts and positive control aspirin on the acetic acid induced writhing response in mice are given in table1. It was found that the bark extracts failed to respond analgesic activity even at high dose 500 mg/kg with respect to control group of animals that received 3-ml/ kg (5%) tween 80 suspensions.

Table 1: Effect of *R mucronata* on writhing response in mice

Treatment	Dose	Number of writhing
Control	3 ml/kg	16.33 ± 0.21
Aspirin	50 mg/kg	1.83 ± 0.16**
RMC extract	250 mg/kg	15.66 ± 0.21
RMC extract	500 mg/kg	16.83 ± 0.10
RME extract	250 mg/kg	16.83 ± 0.15
RME extract	500 mg/kg	15.71 ± 0.58
RMM extract	250 mg/kg	15.83 ± 0.27
RMM extract	500 mg/kg	16.35 ± 0.55
RMW extract	250 mg/kg	15.46 ± 0.17
RMW extract	500 mg/kg	16.22 ± 0.12

**p<0.01, n=6, mean ± S.E.M

Eddy's hot plate method

Table no 2 depicts the analgesic activity of successive solvent extracts of R mucronatat bark. The solvent extracts at low and high dose of 250 and 500 mg/kg failed to produce any statistical significant analgesic activity.

Table no 2: analgesic effect of R mucronata bark extracts in eddy's hot plate method

Treatment	Dose (mg/kg)	0 h	1/2 h	1 h	2 h	3 h	5h
Control	3ml/kg	3.66 ±0.21	3.66 ± 0.21	3.83 ± 0.30	3.66 ± 0.21	3.66 ± 0.21	2.83 ± 2.83
Tremadazole	5	3.50 ± 0.34	7.16 ± 0.60**	7.66 ± 42**	7.83 ±0.30**	7.83 ± .47**	7.50 ± 0.56**
RMC extract	250	3.33 ± 0.42	2.83 ± 0.40	3.0 ± 0.63	2.16 ± 0.47	2.33 ± 0.21	2.00 ± 0.25
RMC extract	500	5.33 ± 0.36	4.16 ± 0.72	3.6 ± 0.58	3.50 ± 0.42	3.00 ± 0.25	2.75 ± 0.41
RME extract	250	4 .00± 0.36	4.66 ± 0.66	3.0 ± 0.63	2.66 ± 0.33	2.66 ± 0.33	2.08 ± 0.41
RME extract	500	4.6 ±0.86	4.5 ± 0.61	4.3 ± 0.55	4.33 ± 0.57	4.16 ± 0.47	2.48 ± .021
RMM extract	250	4.33 ± 0.57	3.5 ± 0.22	2.8 ± 0.49	2.50 ± 0.34	2.66 ± 0.33	1.93 ± 0.08
RMM extract	500	4.66 ± 0.86	2.66 ± 0.49	2.6 ± 0.34	2.80 ± 0.40	2.33 ± 0.21	3.00 ± 0.22
RMW extract	250	4.5 ± 0.56	2.50 ± 0.34	2.5 ± 0.34	2.16 ± 0.30	2.50 ± 0.34	1.86 ± 0.12
RMW extract	500	5.16 ± 0.40	2.8 ± 0.40	3.1 ± 0.30	2.60 ± 0.33	3.30 ± 0.45	2.63 ± 0.27

*p<0.01, *p< 0.05, n=6 mean reaction time in seconds ± SEM

Paw edema method

The results of formalin induced rat paw edema are presented in the table 3. It was found that methanol extract of RMB at 250 and 500 mg/kg significantly inhibited the paw edema in rats at 24 h of the treatment with respect to the control group of animals; while the other solvent of RMB extract showed statically significant activity up to 4 h interval of the extract administration.

Table 3: Anti-inflammatory activity of *R mucronata* bark extracts on formalin induced rat paw oedema
*p<0.05, **p<0.01, n =6, Mean increase in paw volume in ml at different time (h) ±S.E.M

Treatment	Mean increase in paw volume in ml at different time (h) ± S.E.M						
	Dose	**1h**	**2h**	**3h**	**4h**	**5h**	**24h**
Control tween 80(5%)	5 ml/kg	1.28 ± 0.04	2.05 ± 0.022	2.06 ± .021	2.06 ± 0.02	2.075 ± 0.23	2 ± 0.00
Dicofenac	10 mg/kg	0.733 ± 0.02**	0.66 ± 0.021**	0.56 ± 0.02**	0.57 ± 0.03**	0.50 ± 0.00**	0.5±0.00**
RMC extract	250 mg/kg	1.16 ± 0.06	2.033 ± 0.023	1.86 ± 0.033	1.86 ± 0.033	2.11 ± 0.03	2.06 ± 0.026
RMC extract	500 mg/kg	1.08 ± 0.016*	1.68 ± 0.04*	1.70 ± 0.027*	1.70 ± 0.017*	1.82 ± 0.03	1.98 ± 0.042
RME extract	250 mg/kg	1.20 ± 4.48	2.06 ± 0.01	1.86 ± 0.024	1.86 ± 0.024	1.86 ± 0.024	2 ± 0.00
RME extract	500 mg/kg	1.10 ± 0.01*	1.70 ± 0.03*	1.70 ± 0.02*	1.70 ± .300*	2.08 ± 0.016	2.08 ± 0.016
RMM extract	250 mg/kg	1.06 ± 0.021**	1.43 ± 0.23**	1.43 ± 0.230**	1.43 ± 0.230**	1.43 ± 0.230**	2 ± 0.00
RMM extract	500 mg/kg	1.03 ± 0.03**	1.06 ± 0.03**	1.06 ± 0.036**	1.06 ± 0.036**	1.06 ± 0.036**	1.055 ± 0.074*
RMW extract	250 mg/kg	1.83 ± 0.016	2.10 ± 0.14	2.10 ± 0.044	2.10 ± 0.044	2.10 ± 0.044	2.10 ± 0.04
RMW extract	500 mg/kg	1.12 ± 0.16*	1.08 ± 0.04**	1.72 ± 0.033*	1.72 ± 0.033*	1.72 ± 0.033	2.05 ± 0.02

Sponge implantation model

The sub acute model, results are depicted Table 4 animals treated with of RMB extracts at 500 and 250 mg/kg the methanol extract showed dose dependent significant reduction (p<0.01) in granuloma tissue formation; animals treated with aqueous extract at 500 mg/kg showed statically significant (p<0.05) activity.

Table 4: Effects of *R mucronata* bark extracts on sponge pellet induced granuloma in rats

Treatment	**Dose (mg/kg)**	**Mean wet weight in g ± S.E M**	**Mean dry weight in g± S.E.M**
Control(5 % tween 80)	5 ml/kg	511.6 511.66 ± 58.38	164.33 ± 5.09
Diclofenac	10	265.33 ± 24.96**	36.83 ± 2.33**
RMC extract	250	525.33 ± 9.69	157.16 ± 13.75
RMC extract	500	387.83 ± 17.57*	151.83 ± 3.70
RME extract	250	527.66 ± 20.86	185.66 ± 9.49
RME extract	500	374.83 ± 15.42*	179.83 ± 8.81
RMM extract	250	390.33 ± 37.35*	78.66 ± 1.40**
RMM extract	500	201.66 ± 5.57**	41.66 ± 1.97**
RMW extract	250	430.33 ± 12.085	159.83 ± 5.108
RMW extract	500	375.5 ± 40.05*	58.5 ± 2.23*

*p<0.05, **p<0.01,n =6, Mean increase in paw volume in ml at different time (h) ±S.E.M

Adjuvant induced arthritis – immunological method

Methanol extract of R mucronata bark exhibited anti- arthritic activity in dose dependent manner. At a dose of 250 mg/kg and 500 mg/kg significant activity ($p<0.01$) was seen on 14th day of the treatment; while statistically significant ($p<0.05$) activity at 500 mg/kg was seen was seen on 28 th day of the methanol extract treatment. The RMW extract showed significant activity ($p<0.01$)was observed on the 14 th day of the treatment. The results of the chronic inflammation (immunological model) are shown in Table 5.

Table 5 - Anti-inflammatory effect of R mucronata bark extracts against Freud's adjuvant induced paw edema in rats

Treatment	Mean increase in paw volume at different days ± S.E.M				
	Dose	1 day	7 day	14 day	28 day
Control	5 ml/kg	2.13 ± 0.033	2.11 ± 0.042	2.25 ± 0.024	1.93 ± 0.021
Dicolfenac	10mg/kg	2.25 ± 0.022	2.15 ± 0.050	0.85 ± 0.022**	0.53 ± 0.022**
RMC extract	250 mg/kg	2.31 ± 0.100	2.34 ± 0.083	2.26 ± 0.107	2.05 ± 0.03
RMC extract	500 mg/kg	2.33 ± 0.042	2.30 ±0.063	1.883 ± 0.030	1.91 ±0.040
RME extract	250 mg/kg	2.33 ±0.073	2.26 ± 0.084	2.26 ± 0.086	2.26 ± 0.080
RME extract	500 mg/kg	1.14 ± 0.227	1.12 ± 0.220	1.11 ± 0.215	1.09 ± 0.209
RMM Extract	250 mg/kg	1.16 ±0.239	1.15 ± 0.236	0.94 ± 0.199**	1.09 ± 0.202
RMM extract	500 mg/kg	1.13 ±0.260	1.12 ± 0.258	1.09 ± 0.106**	0.92 ± 0.193*
RMW extract	250 mg/kg	1.14 ± 0.262	1.11 ± 0.261	1.96 ± 0.106	2.00 ± 0.106
RMW extract	500 mg/kg	2.41 ±0.054	2.32 ± 0.054	1.07 ± 0.249**	1.00 ± 0.224

*p<0.05, **p<0.01,n =6, Mean increase in paw volume in ml at different time (h) ±S.E.M

Discussion

Inflammation constitutes the body's response to injury and is characterized by a series of events that includes the inflammatory reaction, a sensory response perceived as pain, and a repair process **(12)**. Some causes of an inflammatory reaction are infection, trauma penetrating injury, blunt trauma, thermal injury, chemical injury, and immunologically mediated injury (humoral or cellular) and as a result of the loss of blood supply (ischemia) **(13)**. Inflammation may be acute and chronic **(14)**. Inflammatory response occurs in three distinct phases. The first phase is caused by an increased in vascular permeability resulting in exudation of fluids from the blood into the interstitial space, the second phase involves the infiltrations of leukocytes from the blood into the tissue and in third phase Granuloma formation and tissue repair. Mediators of inflammation originate either from plasma (e.g. complement proteins ,Kinins) or from cells (e.g. histamine, prostaglandins, cytokines). The production of active mediators is triggered by microbial products or by host proteins, such as proteins of the complement, kinins and coagulation systems that are themselves are activated by microbes and damaged tissues. Generally the mediators of inflammation are Histamine, Prostaglandins (PGs), Leukotrienes (LTB_4), Nitric oxide (NO), Platelet-activation factor (PAF), Bradykinin, Serotonin, Lipoxins**,** Cytokines, Growth Factors **(15).**

The most widely used primary test to screen new anti inflammatory agents measures the ability of compound to reduce local edema induced in the rat paw by injection of an irritant agent **(16)**. This edema depends on the participation of kinins and polymorphonuclear leukocytes with their pro-inflammatory factors including prostaglandins **(17)**. The results of our present study indicate the methanol extract of R mucronata bark inhibits acute and sub acute inflammation. The anti inflammatory activity of the extract of RMB can be attributed to the combination of the following mechanism. The development of edema in the paw of the rat after the injection of formalin is biphasic event **(18**) is inhibited; the initial phase, observed around 1 h, which is attributed to the release of histamine and serotonin; the second, accelerating phase of swelling is due to the release of prostaglandin-like substances. And inhibition of proliferate phase of the inflammation of the microphages, neutrophils, fibroblasts and collagen formation which are basic source for the Granuloma formation; therefore decrease in the Granuloma formation indicates the suppression of the proliferate phase. The methanol extracts of RMB showed strong inhibition on the paw edema in the early phase and late phase of the inflammation, implying that extract exert the anti-inflammatory effect by acting on the both phase of the inflammation. The chloroform, ethyl acetate and aqueous extract of RMB showed a weak anti-inflammatory effect, in sub acute inflammation model. This might be due to the decrease in activity of these extracts, which might result from the elimination of extracts, as well as the possible antagonist effects between individual components present in the respective extracts, which induced different patterns of the overall effect. The early inflammatory response mediated mainly by histamine and release of prostaglandins, protease and lysosome **(15, 18)** of the second phase were inhibited the granular formation and tissue repair of the third phase of inflammation was strongly inhibited by the methanol and aqueous extracts of RMB. The positive results from methanol extract can be attributed to the flavanoid, phenol acids, sterols,glycosides and triterpenoids present in the extract as shown in the phytochemical preliminary investigation.

From the foregoing observations for the anti inflammatory and analgesic activity extracts of Rhizophora mucronata bark extracts, it can be concluded- all the solvent extracts of Rhizophora *mucronata* bark failed to show statically significant analgesic activity in acetic acid induced writhing method and Edyy's Hot plate method at 250 mg/kg and at high dose of 500 mg/kg in mice. The methanol extract at a dose 250 and 500 mg/kg (b.w) possess significant anti-inflammatory activity in formalin induced paw edema and sub acute sponge pellet induced inflammation and chronic adjuvant induced arthritis – immunological method in rats. Therefore, the study supports the traditional claim of anti-inflammatory activity of Rhizophora *mucronata*.

References

1. Bandaranayake W M. Traditional and medicinal uses of mangroves Mangroves and Salt Marshes Sept 1998; 2

2. Bandarnayake W M Bioactivities, bioactive compounds and chemical constituents of mangrove plants. Wetlands Ecol. Manage. 2002;10: 421-452.

3. Anjaneyulu A S, Anjaneyulyu V, Rao V L. New beyerane and isoprimarane diterpenoids from Rhizophora mucronata. J Asian Nat Prod Res 2002 mar; 4(1):53-61.

4. Premanathan M, Kathirasan K, Yamamoto N, Nakashima H. Invitro antihuman immuno deficiency virus activity of polysaccharides from Rhizophora mucronata poir. Biosci Biotecnol Biochem 1999; 63(7): 1187-91.

5. Health Effects Test Guidelines. Acute Oral Toxicity. [Computer program] OPPTS 870.1100 United States Office of Prevention, Pesticides and Toxic Substances Environmental Protection Agency (7101). [Available from: URL: http://www.epa.gov/opptsfrs/home/guidelin.htm. 5/6/2004

6. Taesotika T, P anthong A, Kanjanapothi D, Verpoorte R, Scheffer JJC. Antiinflammatory, antipyretic and antinociceptive activities of Tabernaemontana Pandacaqui poir.J Ethno Pharm.2003; 8: 31-35

7. Hajre SW ,Suresh Chabndra, Tandan SK, SarmaJ,Lal J,Telang AG.Analgesic and antipyretic of Dalbergia Sissoo Leaves. Ind J Pharm. 2000; 32: 357-***360***

8. Brownlee G. Effect of deoxocortisone and ascorbic acid on formaldehyde induced arthiritis in normal and adrenalectomized Rats. Lancet. 1950; 1:157-159

9. Finney RSH,Somers GF.The anti-inflammatory activity of Glycerrhetuc acid and derivatives. Department of Biology, Leicester College of Technology and Department of Pharmacol.1958; 613- 619

10. Patwardhan MD, Muley MP, Manekar MS.Anti-inflammatory property of ranitidine, a specific H_2- receptor antagonist. Ind J physiol pharmacy.1958; 30:206-208.

11. RosaM Perez G, Cauauhtemoc Perez G,Miguel A,Z Avulas.Anti-inflammatory activity of Cisus trifoliate. Fitotherapia.1993;2:103-107

12. Mohan H. Inflammation and Healing. Textbook of Pathology, Ed Jaypee Publication, New Delhi 2002, 114-121.

13. Robbins and Cortran,. Acute and chronic inflammation. Pathologic Basis of Disease, Elsevier Publication, Ed 7, 2004, 47-87.

14. Winter, C.A., Risley, E.A. and Nuss, G.W. Carrageenan-Induced Oedema in Hind Paw of Rat as an Assay or Anti-Inflammatory Drugs. Proc. Soc. Exp. Biol. Med 1962; 111**:** 544-547.

15. Dray A. Inflammatory mediators of pain. British Journal of Anesthesia 1995; 25-131.

16. Crunkhon P, Meacock S, Mediators of the inflammation induced in the rat paw by carrageenan. British Journal of Pharmacology 1971; 392-402

17. Damas, J., Remacle-Volon, G and Deflandre, E. Further Studies of the Mechanism of Counter rritation by Turpentine. Arch. Pharmacol. 1986; 332**:**196-200.

18. Vinegar R, Schreiber W, Hugo R. Biphasic development of carrageenan oedema in rats, Journal of Pharmacological Experimental Therapeutics,1969; 96-103

19. Vinegar R, Schreiber W, Hugo R. Biphasic development of carrageenan oedema in rats. Journal of Pharmacological Experimental Therapeutics, 1969; 96-103.