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Prostaglandins and Related Substances

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Prostaglandins and related substances are in prominence again. Discovered over half a century ago, these compounds have attracted the attention of scientists all over the world. Research communications on these substances, which were annually one or two in the nineteen thirties, became so large that a Journal totally devoted to prostaglandins was launched in 1964. The leading workers in this field have been conferred the Nobel Prize for Physiology and Medicine 1982. The present article reviews the historical aspects of prostaglandin research.

Kurzok and Lieb (1930) while attempting artificial insemination in infertile women, observed increased uterine activity, and sperm rejection in a number of cases. In order to understand the basis of this activity they investigated the effects of sperm on uterine stripes *in vitro*. Their work revealed the presence of a dialysable component in semen which was responsible for contraction, and that spermatozoa were not essential for the reaction. Most unfortunately they used these results to explain infertility and postulated an erroneous classification of uteri and spermatic fluid.

Goldblatt (1933) made strikingly similar observations, but used the data to suggest the presence of a smooth muscle contractile component of human seminal plasma, and postulated that this

plasma could have other functions unconcerned with those related to spermatozoal nutrition. If he was aware of the work of Kurzok and Lieb (1930), he gave no indication of the same.

von Euler working in Stockholm and quite unaware of the work of Goldblatt published identical data using whole spermatic fluid (von Euler 1934). Goldblatt however followed up his own observations, and demonstrated hypotensive and spasmogenic effects of seminal plasma and its alcohol extracts, he also demonstrated that the active principle was not acetylcholine, or any other choline ester or histamine (Goldblatt 1935). von Euler extracted a hypotensive substance from vesicular gland (vesiglandin) (von Euler 1935 a) and established its identity with the prostate gland extracts (von Euler 1935 b).

It is curious that the biologically active principle came to be named prostaglandin (von Euler 1935 b) since Goldblatt (1934) had demonstrated that it is not elaborated in the prostate. The name however stuck on, even today the name continues though definitive proof of its occurrence and origin was produced by Eliasson (1959).

The work of von Euler (1935 b) demonstrated that prostaglandin was a fatty acid, and unstable in nature. He however converted it into a stable Ba-

rium salt, which was used as a reference standard for years to come (von Euler 1936). Working with this crude substance evidence was obtained to suggest that prostaglandin contained no nitrogen, but contained at least one hydroxyl group and probably a number of double bonds (von Euler 1939).

Using the barium salt of crude prostaglandins extracted from sheep vesicular glands obtained during the annual slaughter of Icelandic sheep, Bergström prepared a highly purified prostaglandin (Bergström 1949). This purified fraction was used for structural elucidation of prostaglandin (Bergström and Sjövall 1957). The prostaglandin named prostaglandin F (PGF) was found to be potent smooth muscle stimulant, but surprisingly lacked hypotensive effects. The workers surmised that what they had isolated, was just one of the probably numerous compounds from the original extracts. Their subsequent work proved the surmise correct and they shortly announced the isolation and the structure of the second prostaglandin — PGE (Bergström and Sjövall 1960).

Mainly using sodium borohydride, it was demonstrated that PGE could be converted into PGF having various degrees of unsaturation, this led to the realisation that PGE or PGF was a group of substances, which were present as monoene, diene or trienes.

In preliminary biological studies prostaglandins were found to be more potent than any known biological substance, and were ubiquitous in their distribution (Bergström *et al* 1969). These, thus generated immense interest and work on their biosynthesis, function and metabo-

lism began. Since these were fatty acids, it was believed that they could be formed from polyunsaturated fatty acids like arachidonic acid or γ — homo linoleic acid, cleaved off from triglycerides or phospholipids. Using tritiated arachidonic acid Anggard and Samuelsson (1965 a) demonstrated the formation of PGE and PGF by guinea pig lung homogenates. Subsequently Samuelsson and his coworkers delineated the entire biosynthetic pathway for the biosynthesis of prostaglandins and postulated the presence of unstable endoperoxides, as well as the metabolism of prostaglandins into the 15-keto and 15-keto dihydro derivatives in the lung (Anggard and Samuelsson 1965 b).

The English group of workers led by Prof. John R. Vane had by then developed a suitable bioassay for prostaglandins, and established the cascade superfusion for differential estimation of bioactive substances. They also indentified a highly unstable Rabbit Aorta Contracting Substance (RCS) from anaphylactic guinea pig lungs (Piper and Vane 1969). An important contribution of Vane was the demonstration of the inhibition of prostaglandin synthesis by aspirin and other non-steroidal anti-inflammatory agents, (Vane 1971). Using highly sophisticated technology, RCS was fractionated and Thromboxane A₂, Prostacyclin and PGH₂ isolated (Moncada and Vane 1978).

Recent studies on Thromboxane and Prostacyclin suggest that these unstable substances could play a major role in the maintenance of various physiological functions, this role had been in the past attributed to the opposing effects of

PGE and PGF. The prostaglandins have now been suggested to play a variety of roles, including maintenance of smooth muscle tone (Horton and Main 1963), inflammation (Willis 1969), anaphylaxis (Piper and Vane 1969) tumour biochemistry (Sandler et al 1969) parturition (Karim 1969), central synaptic transmission (Horton and Main 1969) hemostasis and platelet function (Moncada and Vane 1978) and autonomic neurotransmission (Hedqvist 1977).

The Slow Reacting Substance of Anaphylaxis (SRS-A) another derivative of arachidonic acid metabolism has an important role in human anaphylaxis and asthma. First reported in 1938, the substance eluded purification and struc-

tural elucidation. A team of chemists working under the leadership of Prof. Bengt Samuelsson in Stockholm succeeded in purifying and elucidating its structure. They reported the existence of an hitherto unknown chemical configuration for which they introduced the nomenclature — Leukotrienes.

The Nobel Foundation in recognition of the work of Prof. John Vane, Prof. Sune Bergström and Prof. Bengt Samuelsson has declared them, joint recipients of the Nobel Award for Medicine and Physiology for the year 1982. The three scientists have collaborated in the past on various aspects of prostaglandin research and have jointly won the Albert Lasker Medical Research Award in 1977.

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Mycobacterial Antigens - a review

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Knowledge of antigenic structure of mycobacteria is important for many reasons. Antigens showing a high degree of polymorphism can be employed to study taxonomic relationship amongst mycobacteria. Runyon's classification (1), based on biotyping, taking products and functions of a number of genes into consideration, does have its limitations. As a result one comes across a number of strains having characteristics not fitting exactly into a particular biotype. Knowledge of taxonomic relationships based on antigenic structure of mycobacteria is most ideal, because it will have important implications on issues such as cross-protection, natural immunity acquired as a result of infection with environmental mycobacteria having low or no virulence, vaccine development, and interpretation of epidemiological data. Reported failure of BCG (2) as well as attempts to develop new mycobacterial vaccines employing environmental avirulent strains, bring the issue of antigenic relationships of mycobacteria into focus.

Tuberculin and PPD have been widely used for diagnostic purposes. These are mixtures of antigens (3, 4, 5, 6) many of which have extensive cross-reactions (7).

In this review, a summary of significant information on mycobacterial anti-

gens will be presented. Mycobacterial extracts contain many biologically active substances such as adjuvants. These will not be considered here.

Historical background :

The study of mycobacterial antigens dates back to 1890 when Robert Koch reported the "Old tuberculin" (8). Florence Seibert (9, 10) attempted to purify "tuberculin" by trichloroacetic acid (TCA) precipitation and she developed a preparation known as "purified protein derivative" (PPD). Since then PPD has remained the standard preparation. Affronti (11) prepared PPDs from different mycobacteria, attempted epidemiological studies with them and experienced the problem of extensive cross reactions. Seibert (12) fractionated the "tuberculin" by alcohol and acetic acid precipitation. She described four proteins A to D and two polysaccharides. Proteins A and B could elicit delayed hypersensitivity (DH) response.

Electrophoretic and Immuno-electrophoretic profiles :

Mycobacterial cell extracts can be prepared by disrupting washed whole bacterial cells by a variety of methods such as grinding, pressure rupture and sonication. The lysate can be freed from particulate matter by centrifugation.

Polyacrylamide gel 'disc' electrophoretic profile (at pH 9.5) of such a sonicate reveals a number of bands throughout the gel, even though separation is not very good. The bands are not sharp and there is considerable blurring and trailing. Considerable amount of protein is seen at the interface of stacking and separation gels (13). Discontinuous gels having decreasing pore size and gradient gels can considerably improve the resolution of the sonicate. If the sonicate is electrophoresed on agarose gels one can not get discrete bands (13).

Single and two dimensional immunoelectrophoresis has been employed to study the pattern of antigens in mycobacterial extracts and products (14, 15). Employing antisera prepared against unheated culture filtrates and mycobacterial cell extracts, Daniel *et al* (16, 17) analysed the antigenic components by immunoelectrophoresis. Precipitation arcs obtained were numbered 1 to 10. Employing two dimensional immunoelectrophoresis Roberts *et al* (15) have characterised 41 different components in the mycobacterial sonicate.

One may think of employing the above two techniques for nomenclature of mycobacterial antigens. For example the antigen 5 a precipitin arc in the immunoelectrophoretic profile of culture filtrate has been characterised by Daniel *et al* (7, 18, 19) to be associated with skin reactivity to tuberculin, lymphocyte mitogenicity and macrophage migration inhibition activity. This antigen has an Rf value of 0.61 on 7.5% polyacrylamide gel at pH 9.5 (20). However these techniques have gross limitations. On polyacrylamide gel, separation of the antigenic components is not very satisfactory.

Immunoelectrophoresis as a procedure in the study of these antigens also has limitations. The precipitin arcs are attenuated and often variable, the pattern being quite sensitive to variations in culture conditions, immunisation protocols and conditions of immunoelectrophoresis (17). Many antigens of the culture filtrates and cell extracts are derived from cell walls where repeating units abound; as a result the individual antigenic determinants may be present on fragments of various sizes, carrying different charges. Ion exchange chromatographic studies of Knicker (21, 22) confirm this view.

Chemistry: The antigenic preparations of mycobacteria, such as culture filtrates and cell extracts prepared by sonic disintegration contain proteins, polysaccharides and lipids. Polyacrylamide gel 'disc' electrophoresis has been employed to study proteins. Most polysaccharides do not enter the gels, they are held at the interface of separation and stacking gels (13); lipids tend to form micelle and hence do not enter the separation gel freely. Since, in immunoelectrophoresis one can only characterise antigens in terms of precipitin arcs of macromolecules of differing Rf values, chemical nature of each antigen is not evident.

Employing ethanol acetic acid precipitation Seibert (12) characterised four proteins A, B, C & D and two polysaccharides I & II; none of these were antigenically pure. Knicker and La Borde (22) employed ion-exchange chromatography to obtain protein and polysaccharide rich fragments; none of which were antigenically pure. Baer and Chaparas (23, 24, 25) carried out molecular exclusion chromatography to obtain enriched fractions with protein and polysaccharide

antigens none of which were pure. Employing gradient acrylamide gel electrophoresis, Minden and Farr (26) could isolate a rapidly migrating glycoprotein with a molecular weight of 9000-12000 daltons, soluble in 50% saturated ammonium sulphate. It appears to be species specific. Daniel and his associates (20, 27, 28, 29) have employed concanavilin A and immunoabsorbent affinity chromatography to isolate arabinogalactan, arabinomannan as well as antigen 5 and antigen 6 in a highly purified form. Many investigators have employed two or more physicochemical techniques in succession to obtain pure antigens.

Despite the use of these techniques singly or in combination, the characterisation of antigens still remains descriptive and the reproducibility of physicochemical characters of any particular antigen is always subject to the particular isolation procedure employed. Deshpande *et al* (13) have recently presented reasons to explain some of the difficulties in separation and characterisation of mycobacterial antigens. The mycobacterial cell wall is rich in lipids and as a result antigens of the outer membrane and cell wall are likely to remain in association with the lipids. They have presented evidence to indicate that the majority of protein antigens in the sonicate of *M. tuberculosis* H37Rv are lipoproteins and the apoproteins are extremely hydrophobic. As a result, separation of such antigens in aqueous milieu, employing techniques such as gel filtration and ion exchange chromatography is very difficult. These investigators have employed proteolytic digestion followed by repeated chromatography on sephadex G 200 to characterise an antigenic material 150,000

daltons, consisting of protein and free of lipid. This antigen capable of eliciting DH response, has electrophoretic mobility and m. wt. quite different from antigen 5, described by Daniel and Anderson (20). Kuwabara and Tsumita (30) have isolated a peptide from *M. tuberculosis*, having m. wt. of 9700 daltons and characterised its primary structure. This material has been crystallised. It can elicit DH response.

The polysaccharide antigens have been studied in great details. Misaki, Azuma, and Yamamura 31-36 have characterised four types of protein free polysaccharides namely arabinogalactan, arabinomannan, mannan and glucan. The detailed structural formulae of the first three have been described (33, 34, 35). Arabinogalactan and arabinomannan in general consist of a central core with oligosaccharide side chains formed of arabinose and/or mannose. There is a high degree of chemical and antigenic similarity amongst polysaccharides isolated from various mycobacteria. Even nocardia and corynebacteria possess polysaccharides similar to those of mycobacteria (37).

Specificity: As in all other bacteria, mycobacterial antigens show extensive cross-reactions. When one takes into account a large number of antigens present in the cell extract the issue of specificity becomes highly complex. Major hurdles in the study of mycobacterial antigens are, the inadequate knowledge of physicochemical characteristics and difficulty in isolation of pure antigen. In particular the following two issues are of prime importance: (1) Are there any unique antigens, characteristic of groups

and species ? (2) What is the degree of polymorphism of a well characterised antigen ? Are there distinct common and species specific determinants which can be employed as serological markers ? Both these issues can only be handled successfully provided chemistry of the antigens is well understood. Attempts so far to prepare antisera with these aims have had set backs (7).

The polysaccharide antigens show extensive cross reactivity not only with nocardia and corynebacteria but even with staphylococci (38). In contrast at least some proteins can be specific ; but the data is preliminary. Antigen 5 of Daniel (20) has been claimed to be limited to *M. tuberculosis* and *M. bovis*,

Work of Minden and Farr (26) and Roszman and his associates (39) indicates that relatively low m. wt. antigens are likely to be species specific. However one has many reservations in accepting this. A highly polymorphous large m. wt. antigen can have on the same molecule species specific as well as non specific common antigenic determinants. Low m. wt. antigens described earlier could very well be fragments of such large m. wt. molecules. This appears to be a likely possibility because the reproducibility of chemical and immunological characters of a macromolecule remains subject to following a particular isolation protocol.

Standford and his associates (40, 41) have analysed the precipitin arcs developed by immunodiffusion employing antisera against mycobacterial cell extract. The antigens have been divided into four groups. The group (i) antigens are shared by mycobacteria, nocardia and corynebacteria. Group (ii) and (iii)

antigens are characteristic of slow and fast growing mycobacteria respectively. The antigens of group (iv) are species specific. However purified monospecific antisera have so far not been prepared. A consistent, reproducible method of serotyping is still nowhere in picture.

Immunobiology :

Knowledge of association of a particular antigenic activity with a given protein or polysaccharide is very much essential. For example identification of antigen responsible for cellular immunity would facilitate rational study of problems such as cross-reactivity, immune-suppression and deviation and immunity acquired as a result of infection with avirulent environmental mycobacteria.

One of the major problems in correlation of immunological characteristics with physicochemical features of macromolecules is the molecular heterogeneity. As pointed out earlier the same antigenic determinants could very well be present on fragments of varying sizes carrying different charges.

Association of antigen 5 of Daniel *et al* with various cell mediated immune reactions has already been alluded to earlier. Daniel and Affronti (16) have also shown that protein A & B of PPD contain antigen 5. The antigen fractions capable of eliciting DH, as reported by Baer and Chaparas (20) and Moulton (42) appear to be consisting of many components. Gupta and Landi (43) have isolated a DH eliciting peptidoglycan of 28-30,000 dalton m. wt. Primary structure of the DH eliciting unit of the crystallisable peptide reported by Kuwabara and Tsumita (30) has been determined to be Asn-Gly-Ser-Glu-Met-Arg. Deshpande *et al* (13) have reported a 150,000 dalton DH eliciting

lipoprotein. It is very difficult to compare the DH eliciting antigens reported by various investigators because of reasons already discussed earlier. One of the logical approaches to this problem is to raise marker antibodies to each of the purified antigens. The reactivity of the various antigens against marker antisera can then be compared.

Arabinogalactan and arabinomannan can elicit immediate reactions as well as passive cutaneous anaphylaxis. Highly purified polysaccharides can not elicit delayed hypersensitivity responses. However one can not rule out the possibility of oligosaccharide residues on glycoproteins participating in cell mediated immune reactions.

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Studies of Anaphylaxis IV. Comparison of 48/80 and X-537A induced mouse paw oedema

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Development of a new model for drug screening, involves demonstration of the specificity of the model and effects of standard drugs. Compound 48/80 has been demonstrated to induced mouse paw oedema and this model has been suggested as a preliminary screen for antianaphylactic agents. The oedema has been compared with that induced by a non-specific ation ionophore, with respect to calcium dependence and inhibition by standard antianaphylactic agents. Results indicate that antianaphylactic agents specifically inhibit 48/80 induced oedema without affecting X 537 A induced reactions.

Introduction

Agents which inhibit the release of mediators of anaphylaxis, induced by antigens, are clinically useful in reducing the frequency and intensity of asthmatic attacks (Cox *et al*, 1971). Compounds like 48/80 and ionophore A 23187, also cause the release of mediators from sensitised or non-sensitised tissues, by mechanism which are similar to those employed by antigens (Gomperts 1976). Studies using the prototype antiasthmatic agent — disodium cromoglycate suggest that it could inhibit mediator release induced by A 23187 (Johnson and Bach 1975) and 48/80 (Foreman and Garland 1976).

Compound 48/80 on intrapedal administration, has been demonstrated to cause a local release of mediators, leading to the formation of a rapidly developing oedema (Ghooi *et al* 1980). It has been demonstrated that histamine plays a major role in the reaction, which is

sensitive to changes in extracellular calcium ions and available metabolic energy. On this basis it was suggested that the model could be used for preliminary screening of antianaphylactic agents (Ghooi and Bhide 1981).

A large number of models are presently available for the screening of antianaphylactic agents, which use antigens or auto-coid liberators to induce mediator release. Most of these models have been found to cumbersome, uneconomical for large screening programmes, or both. The mouse paw oedema model is superior to the presently available ones, in its cost and speed of screening.

The present paper deals with further studies on the paw oedema model, using standard inhibitors of mediator release. Oedema itself being induced by, a calcium dependent liberator — 48/80 and a relatively non-specific cation ionophore — X 537 A.

Materials and Methods

Compound 48/80 was purchased from Sigma Chemical Co., U.S.A., it was dissolved in saline (100 mcg/ml) and stored in aliquots of 1.0 ml each, at -20 C. For administration, the aliquot was diluted to obtain a concentration of 6.0 mcg/ml in saline. A fresh aliquot was used for each experiment.

Compound X 537 A was obtained as a gift from M/s. Hoffman LaRoche, Basle. The compound was dissolved in a minimum amount of ethanol, and diluted in saline to a concentration of 40 mcg/ml and stored in 1.0 ml aliquots at -20 C. No aliquot was reused after thawing.

Disodium cromoglycate was obtained from Fison Laboratories Limited, U.K., Isoprenaline sulphate, ethylene diamino-tetra acetic acid disodium salt from B.D.H., Bombay, and Aminophylline from Fair Deal Corporation, Bombay.

Mice of the Kausali strain, were purchased from Haffkine Bio-Pharmaceuticals Corporation Ltd., Bombay, animals of either sex weighing between 18 to 22 G. were used for the study.

Mouse paw measurements were made using a pocket thickness gauge, of Mitutoyo Manufacturing Co., Ltd., Japan. Induction of paw oedema was carried out as described earlier (Ghooi and Bhide 1981). Six animals were used for each set. Statistical significance was calculated using Student's T test.

Results

(i) Compound 48/80 and X 537 A induced a rapidly developing oedema in the mouse paw, the peak of which was observed at 10 min. A linear relationship between the dose and the response was obtained for both compounds at dose ranges shown in Table 1.

Table 1
Dose response of 48/80 and ionophore X 537 A

Compound	Dose in mcg/paw	Oedema Units	± S.E.
48/80	0.078	8.17	± 0.7
	0.15	17.6	± 0.8
	0.30	23.5	± 1.05
X 537 A	0.25	9.0	± 0.65
	0.5	12.17	± 1.22
	1.0	14.83	± 0.86
	2.0	21.17	± 0.87

1.0 Unit of oedema represents an increase in paw thickness by 0.001 inches.
number of animals — 6 per group.

(ii) Ethylenediamine tetracetic acid (EDTA) at a final concentrations of 1.0 mM caused a significant inhibition of 48/80 induced mouse paw oedema but was without any effect on oedema induced by X 537 A. The results are given in Table 2.

Table 2
Effect of EDTA on 48/80 and X 537 A induced oedema

	Compound 48/80		Compound X 537 A	
	Oedema Units	$\pm$ S.E.	Oedema Units	$\pm$ S.E.
Control	20.83	0.62	21.16	$\pm$ 0.87
EDTA 1.0 mM	12.00	—	20.16	$\pm$ 0.54
	P < 0.001		P > 0.1	

Dose of 48/80 — 0.3 mcg/paw

Dose of X 537 A — 2.0 mcg/paw

(iii) Isoprenaline, aminophylline and disodium cromoglycate (DSCG) all inhibited the 48/80 induced oedema, when the compounds were tested by the local route (administered with 48/80). By the intraperitoneal route only DSCG afforded significant protection. Results of the test using drugs by the local route are shown in Table 3, and those in which the intraperitoneal route was employed are shown in Table 4.

Table 3
Effect of drugs on compound X 537A induced paw oedema

Drug	Dose mcg local	Oedema units	S.E.	Inhibition
—	—	20.16	$\pm$ 0.65	—
DSCG	100	21.33	$\pm$ 0.84	—
Isoprenaline	25	19.66	$\pm$ 0.66	—
Aminophylline	500	20.50	$\pm$ 1.05	—
Mepyramine maleate 1.0 mg/kg i.p.		3.16	$\pm$ 0.50	83.12

Dose of X 537 A — 2.0 mcg.

Mepyramine administered 30 min. prior to X 537 A.

Table 4
Inhibition of compound 48/80
induced oedema

Compound	Dose mg/kg	Inhibition
DSCG	0.3	37.0
	1.0	62.5
	3.0	80.0

Oedema induced by 0.3 mcg/paw 48/80.

DSCG administered 15 min. prior to 48/80.

(iv) X 537 A induced oedema was not affected by any of the standard antianaphylactics agents, in doses at which 48/80 induced oedema was inhibited, these results are shown in Table 5.

Table 5
Inhibition of compound 48/80 induced
paw oedema

Compound	Dose mcg/paw local	Percent Inhibition
Isoprenaline	0.1	17.3
	1.0	69.7
	10.0	91.1
DSCG	5.0	17.7
	25.0	27.7
	50.0	75.6
Aminophylline	12.5	—
	125.0	46.9
	500.0	69.9

Oedema induced by 0.3 mcg/paw 48/80

Discussion

Compound 48/80, over a short dose range and compound X 537 A induced a dose dependent paw oedema in mice. The peak of oedema for both agents is at 10 min., which suggests that long acting mediators like Slow Reacting Substance of Anaphylaxis (SRS-A) are probably not involved. While Chakravorty *et al* (1960) suggested that compound 48/80 releases along with histamine a lipid soluble smooth muscle contracting principle, the work of Burka and Flower (1979) demonstrated that 48/80 is a poor liberator of SRS-A.

Reactions induced by 48/80 bear some resemblance to those induced by antigens in sensitised animals or tissues. The present studies indicate that standard antianaphylactic agents cause an inhibition of 48/80 induced paw oedema. The order of potency of these agents on the oedema model is Isoprenaline DSCG Aminophylline, which compares very well that obtained on bronchial lavage cells (Butchers *et al* 1980) leukocytes (Lichtenstein and Margolis 1968), and in the clinic (Pain 1973).

Compound X 537 A also causes the release of mediators and induces paw oedema. Its reaction is however, independent of extracellular calcium ion concentration, and chelation of calcium by EDTA has virtually no effect on the oedema produced. In this respect it differs significantly from compound 48/80. Further while 48/80 induced oedema is inhibited by antianaphylactic agents, oedema induced by X 537 A is not influenced. This observation suggests that inhibition of oedema by agents is not a

non-specific physiological antagonism, and that these agents act probably at the site of calcium involvement in the release process.

The present studies thus reveal that antianaphylactic agents inhibit mouse paw oedema induced by 48/80 but not that induced by X 537 A. The ED 50 of DSCG obtained by this model (0.7 mg/

kg) compares well with the value reported by other workers (Niemegeers *et al* 1978). The order of potency of standard agents on this model also agrees with that reported elsewhere.

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Studies on Anaphylaxis. V: *Butea monosperma* (Palas) as a Potential Antiasthmatic agent

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A fraction (F-X) containing sodium salts of the phenolic constituents of the plant was isolated from the bark of *Butea monosperma* (Palas).

The fraction (F-X) inhibited the formation of compound 48/80 induced as well as antigen induced mouse hind paw oedema and this inhibition was susceptible to blockade by propranolol. The fraction also produced relaxation of bronchi *in vitro* and inhibited histamine constricted bronchi *in vivo* in guinea pigs, which was blocked by propranolol.

Introduction

A number of plants have been used, since times immemorial for the treatment of bronchial asthma. *Butea monosperma* or *B. frondosa* (fam. Leguminosae) (Palas, Palash) have been used in Ayurvedic system of medicine, (Nadkarni, 1976). Murti and Seshadri (1940, 1941, 1944), Kalyesaraj and Kurup (1967), Seshadri and Trikha (1974); have studied the chemistry of different parts of the plant. The plant contains various types of flavanones, chalcones, their polymers and other familiar compounds. Rajapurkar *et al* (1961), Kalyesaraj and Kurup (1967, 1968), have reported an anthelmintic principle from the seeds of the plant. The present investigation was planned to study the pharmacological properties of the bark of *B. monosperma*, with special reference to its bronchodilator and/or anti-anaphylactic activity.

Material and Methods

Phytochemistry;

Bark of plant *B. monosperma* was collected from Vashi, New Bombay, shade dried and coarsely powdered. The powder was serially extracted with petroleum ether, chloroform and methanol in a Soxhlet apparatus. These fractions were subjected to general pharmacological investigations and based on the results of these tests, methanol extract, which showed bronchodilator activity, was used for further purification.

The methanolic extract was completely dried and redissolved in cold methanol, when a part of the extract was found to be insoluble. The soluble and insoluble parts were separated and screened for their activity. The insoluble part was active.

This fraction was extracted with 1 per cent sodium hydroxide solution, when a

dark red solution of acids and phenols was obtained with a small amount of greenish residue. The residue was inactive, hence discarded. To the sodium hydroxide solution, 10 per cent hydrochloric acid was added till a dark brown coloured mass precipitated. This precipitate was filtered, dried and crystallised by redissolving in sodium hydroxide, neutralizing the solution with 10 per cent hydrochloric acid and evaporating to dryness. This salt fraction (F-X) was further used for detailed pharmacological studies. Various analytical and chemical spot tests were employed for determining the nature of the fraction.

Pharmacology :

Compound 49/80 was obtained from Sigma Chemical Co., U.S.A., propranolol and isoprenaline sulphate from I.C.I., U.K., DSCG from Fison Pharmaceutical Laboratories, U.K. and adrenaline from Themis Pharmaceuticals, Bombay. Ovalbumin was prepared by ammonium sulphate precipitation of egg white, and purified by recrystallisation in the laboratory.

Albino mice (N.I.H. or Kasauli strain), guinea pigs (random bred) were obtained from H.B.P.C.L., Bombay. Male mice of C 57BL/6 strain were obtained from Director's Laboratory, of the Haffkine Institute.

Paw thickness measurements were made with a Pocket Thickness Gauge (Mitutoyo Manufacturing Co., Japan).

1. Study of bronchodilator activity :

(a) Isolated tracheal chain preparation of guinea pig : (Castillo and DeBeer, 1947). Guinea pig tracheal chain was

prepared and mounted in an organ bath, containing Kreb's solution gassed with carbogen. Relaxation of the muscle was recorded on a kymograph using isotonic frontal writing lever. Isoprenaline (2-8 ng/ml) and propranolol (5 ng/ml) were used as standard agonist and antagonist respectively. Effects of the fraction (F-X), *per se* or on submaximal doses of isoprenaline were studied (50-1600 mg/ml).

(b) Respiratory resistance in anaesthetised guinea pigs : (Konzett and Rossler, 1940). Guinea pigs of either sex (350-450 gms) were anaesthetised with Chloralose and urethane mixture, i.p. and prepared for record of respiratory resistance as described by Konzett and Rossler (1940). Histamine (1.25-20 ng/ml) was used to induce bronchoconstriction and effect of fraction (F-X) *per se* on the respiratory resistance and histamine induced resistance was studied (Dose 1.25-20 mg/kg).

2. Study of antianaphylactic activity :

(a) Active paw anaphylaxis in mice : (Ghooi and Bhide 1981) Mice of C 57BL/6 strain (male, 20-25 gm) were sensitized with 0.25 mg egg albumin along with aluminium hydroxide gel 6 mg/animal, subcutaneously. After 8 days the animals were challenged with egg albumin as antigen, locally in the hind paw. In the right paw animals received 0.05 ml egg albumin solution (200 ug/ml) in saline while left paw received same volume of saline. The paw thickness of both paws was measured before and 10 minutes after injections by Pocket Thickness Gauge. Oedema produced by antigen-antibody reaction was the difference in increase in thickness, in antigen-injected

and saline injected paws. The thickness gauge is calibrated in division of 0.001 inch each, hence one division was termed as one unit of oedema or thickness.

The effects of fraction (F-X) on production of oedema by i.p. (25-100 mg/kg) and local (5-20 ug/paw) routes and interaction with propranolol (1 mg/kg) were studied.

Anaphylactoid reaction in mice: (Ghooi *et al* 1980)

Mice (either sex, 20-25 gm) were used to induce anaphylactoid oedema with compound 48/80, by the method of Ghooi *et al* 1980. Compound 48/80 in the dose of 0.3 ug in 0.05 ml saline was injected in the right hind paw of the animal and the left paw received equal volume of saline. Anaphylactoid oedema produced by compound 48/80 was calculated as in active paw anaphylaxis. Effects of (F-X) on the reaction by i.p. (25-100 mg/kg) and local (6-24 ug/paw) routes and interaction with propranolol (1 mg/kg) was studied.

In addition Disodium cromoglycate, i.p. (0.3-3.0 mg/kg) local (5-50 ug/paw) and isoprenaline, local (0.1-10 ug/paw) were also studied as standard antianaphylactic agents.

3. Cardiovascular effects :

(a) Isolated guinea pig atria : (Clarke 1926) Isolated atria of guinea pig was mounted in a 10 ml bath with Feigan's solution, gassed with oxygen and contraction were recorded on a kymograph using Starling heart lever. Adrenaline was used as standard agonist to cause an increase in force and rate of contraction. Effects of fraction (F-X) *per se*

and on submaximal adrenaline responses was studied.

(b) Cat blood pressure and respiration : Cats (either sex, 3-4 kg) were anaesthetised with cloralose urethane mixture, i.p. The trachea was cannulated and expired air volume was recorded on a kymograph with a tambour recorder. Blood pressure was recorded simultaneously by cannulating carotid artery through a displacement manometer. The drugs were injected i.v. through jugular vein. Acetylcholine, histamine, adrenaline and isoprenaline were used as standard agents. The effects of fraction (F-X) *per se* and on the standard agents was studied.

4. General Pharmacological Studies :

Fraction (F-X) was also studied for its other pharmacological effects, toxicity, using different *in vitro* and *in vivo* techniques.

(a) *In Vitro* Studies :

(i) Isolated Guinea pig Ileum : Effect of (F-X) in dose range (50-400 ug/ml) *per se* on the preparation or on the standard agonist (histamine and acetylcholine) induced submaximal responses was studied.

(ii) Isolated Rat Uterus : Oxytocin and carbachol were used as standard agonists, fraction (F-X) was studied on the preparation for its *per se* effects or for the interaction (potentiation or blockade) with the submaximal doses of standard agonists.

(iii) Isolated Frog Rectus Abdominis Preparation :

Effect of fraction (F-X) on the preparation *per se* or modification of the

standard agonist (acetylcholine) sub-maximal responses was studied.

(iv) Isolated Rat Phrenic Nerve Diaphragm : (Bulbring 1946)

Fraction (F—X) was studied on the preparation at a single dose (400 μ g/ml) on the contractions of the diaphragm muscle, induced by electrical stimulus; indirectly through nerve or directly.

B. *In Vivo* Studies :

(i) LD₅₀ : Fraction (F-X) was administered to albino mice (Kasauli strain, either sex, 20-25 gms.) in doses ranging from 100—1600 mg/kg i.p. with 10 animals per group. The mortality upto 48 hours and other effects if any, were observed.

(ii) Antiinflammatory Studies : (Winter *et al* 1962) Effect fraction (F-X) in a single dose (400 mg/kg) i.p., (given $\frac{1}{2}$ hour prior) was studied on carrageenan induces rat hind paw oedema by the method of Winter *et al* (1962), with phenylbutazone as standard drug.

(iii) Analgesic Activity :

The tail — clip method of Soaje-Ehaque and Lim (1962) was used with aspirin as standard drug. (F-X) (i.p. 400 mg/kg) $\frac{1}{2}$ hour before experiment, was studied for its analgesic activity.

(iv) Anticonvulsant Activity : (Bianchi and Franceschin 1954)

Effect of fraction (F-X) against leptazol induced convulsions in mice. (either sex, 20-25 gm) in a dose of 400 mg/kg i.p., $\frac{1}{2}$ hour prior to experiment was studied.

Results :

Phytochemistry :

Various analytical and chemical tests used revealed a phenolic carboxylic acid nature or presence of phenolic (-OH) and acidic (-COOH) compounds. The colour tests used to detect flavonoids were positive.

Pharmacology :

1. Study of bronchodilator activity :

(a) Isolated tracheal chain of guinea pig :

Fraction (F-X) in dose range (50 — 200 g/ml) potentiated the submaximal isoprenaline response (2 ng/ml) on bronchial smooth muscle, though it failed to produce any *per se* effects. In higher dose range (400 — 1600 g/ml) a dose dependent relaxation of tracheal smooth muscles was seen. Propranolol (5 ng/ml) blocked the relaxation induced by isoprenaline and fraction (F-X) (Fig-1).

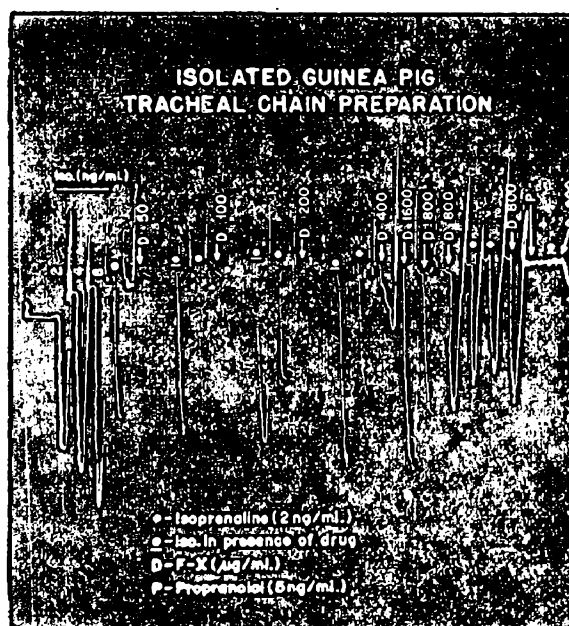


Fig. 1

(b) Respiratory resistance in anaesthetised guinea pigs :

In the dose range (1.25 — 20 mg/kg) fraction (F-X) inhibited histamine induced bronchoconstriction dose dependently (Fig. 2)

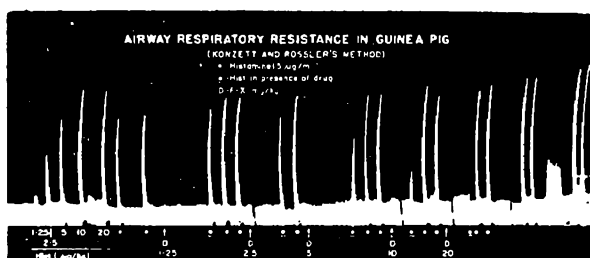


Fig. 2

2. Study of antianaphylactic activity :

(a) Active paw anaphylaxis in mice :

Fraction (F-X), inhibited the oedema produced by active paw anaphylaxis in mice, dose dependently by both routes, local and i.p., in the dose range (5 — 10 μ g/paw) and 25 — 100 mg/kg, respectively. Propranolol (1 mg/kg) i.p., given $\frac{1}{2}$ hour before experiment prevented the inhibition of oedema due to fraction (F-X) by i.p. route. The detailed results are illustrated in table 1 & fig 3.

EFFECT OF F-X ON ACTIVE PAW ANAPHYLAXIS IN MICE

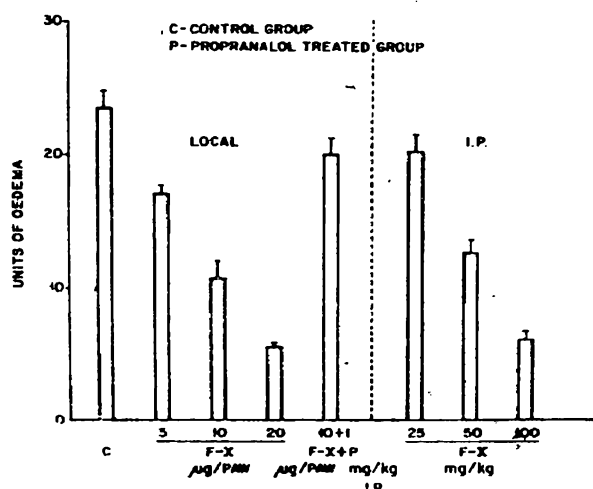


Fig. 3

(b) Anaphylactoid reaction in mice :

Fraction (F-X) local (6 — 24 mg/paw), i.p. (25-100 mg/kg) and i.v. (20 mg/kg) caused a dose dependent inhibition of the compound 48/80 induced oedema in mice hind paw. Propranolol 1 mg/kg prevented the inhibition of oedema due to the fraction (Table 2 and fig. 4).

EFFECT OF F-X ON COMPOUND 48/80 OEDEMA IN MOUSE PAW

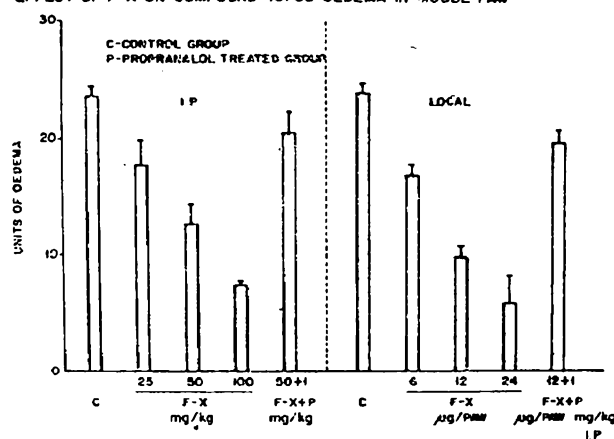


Fig. 4

In study of standard antianaphylactic drugs, DSCG (i.p. & local) and isoprenaline (local) also caused a dose dependent inhibition of the oedema. (Table 3).

3. Cardiovascular Effects :

(a) Isolated Guinea pig atria : Fraction (F-X) upto 800 μ g/ml. did not exhibit any effect on atrial muscle. However, it markedly potentiated the submaximal responses to adrenaline in a dose of 200 μ g/ml and above.

(b) Cat B.P. and Respiration : In a dose range 5-20 mg/kg body weight, fraction (F-X) produced a dose dependent fall in B.P. and increase in respiration.

TABLE 1
Effect of Fraction (F-X) on active paw anaphylaxis

Group	Treatment	Route	Dose	Mean Oedema ± S.E.	% Inhibition	ED ₅₀
1.	Control	—	—	23.6 ± 1.29	—	—
2.	F-X	Local	5 µg/paw	17.2 ± 0.66	27.12	9 µg/paw
3.	F-X	Local	10 µg/paw	10.8 ± 1.24	54.20	
4.	F-X	Local	20 µg/paw	5.6 ± 0.25	76.30	
5.	F-X + P	Local + i. p.	10 µg/paw + 1 mg/kg	20.2 ± 1.15	14.5	
6.	F-X	i. p.	25 mg/kg	20.4 ± 1.22	13.6	55 mg/kg
7.	F-X	i. p.	50 mg/kg	12.8 ± 0.86	45.8	
8.	F-X	i. p.	100 mg/kg	6.2 ± 0.58	73.8	

F-X — (grs 1 to 5 administered along with antigen, grs 6 to 8 administered $\frac{1}{2}$ hour before antigen ; P — Propranolol (gr. 5) $\frac{1}{2}$ hour before antigen.

R.P. — 10 µg/paw egg albumin in 0.05 ml. saline, L.P. — 0.05 ml. saline.

TABLE 2
Effect of Fraction (F-X) on Compound 48/80 Oedema

Group	Treatment	Route	Dose	Mean Oedema ± S.E.	% Inhibition	ED ₅₀
1.	Control	—	—	24.00 ± 0.73	—	—
2.	F-X	Local	6 µg/paw	17.00 ± 0.84	29.2	9.72 µg/paw
3.	F-X	Local	12 µg/paw	10.16 ± 0.83	58.66	
4.	F-X	Local	24 µg/paw	5.83 ± 1.39	75.70	
5.	F-X + P	Local + i.p.	12 µg/paw + 1 mg/kg	19.83 ± 0.83	17.40	
6.	Control	—	—	23.6 ± 0.94	—	—
7.	F-X	i.p.	25 mg/kg	17.83 ± 2.08	25.0	60 mg/kg
8.	F-X	i.p.	50 mg/kg	12.83 ± 1.55	43.64	
9.	F-X	i.p.	100 mg/kg	7.5 ± 0.35	69.64	
10.	F-X + P	Local + i.p.	50 + 1 mg/kg	20.54 ± 1.95	12.97	
11.	F-X	i.v.	20 mg/kg	11.66 ± 1.33	51.40	

F-X — (grs 1 to 5 administered alongwith Compound 48/80, grs 7 to 10 administered $\frac{1}{2}$ hour before compound 48/80,
P — Propranolol (gr. 5) $\frac{1}{2}$ hour before F-X + compound 48/80, gr 10 $\frac{1}{2}$ hour before F-X.

R.P. — 0.3 µg/paw compound 48/80 in 0.05 ml. saline, L.P. — 0.05 ml. saline.

TABLE 3
Effect of DSCG and isoprenaline on Compound 48/80 Oedema

Group	Treatment	Route	Dose	Mean Oedema ± S.E.	% Inhibition	ED ₅₀
1.	Control	—	—	22.5 ± 0.64	—	—
2.	DSCG	Local	5.0 µg/paw	18.3 ± 2.56	18.57	15 µg/paw
3.	DSCG	Local	10.0 µg/paw	16.5 ± 1.28	26.60	
4.	DSCG	Local	20.0 µg/paw	5.5 ± 0.5	75.30	
5.	Isoprenaline	Local	0.1 µg/paw	19.5 ± 1.47	13.30	0.452 µg/paw
6.	Isoprenaline	Local	1.0 µg/paw	6.85 ± 0.91	69.5	
7.	Isoprenaline	Local	10.0 µg/paw	2.00 ± 0.61	91.1	
8.	Control	—	—	20.0 ± 1.80	—	0.77 mg/kg
9.	DSCG	i. p.	0.3 mg/kg	12.6 ± 0.92	37.00	
10.	DSCG	i. p.	1.0 mg/kg	7.5 ± 1.04	62.50	
11.	DSCG	i. p.	3.0 mg/kg	4.0 ± 2.12	80.00	

R.P. — 0.03 µg/paw compound 48/80 in 0.05 ml saline.

L.P. — 0.05 ml. saline.

tion in anaesthetised cat. The effects of acetylcholine, histamine, adrenaline were not significantly affected but isoprenaline responses were slightly potentiated. Propranolol (1 mg/kg) blocked both B.P. and respiratory responses to fraction (F-X).

4. General Pharmacological Studies :

(A) *In Vitro* :

(a) Isolated Guinea Pig Ileum :—

Fraction (F-X), in dose range (100-400 ug/ml), inhibited the submaximal doses of histamine (100 ug/ml) and acetylcholine (200 ng/ml). While histamine inhibition was dose dependent (20-50%), acetylcholine inhibition was not dose dependent (15-20%). Fraction *per se* did not cause any effect.

(b) Isolated rat uterus: Fraction (F-X), (200-800 ug/ml) showed a blockade of carbachol and oxytocin submaximal responses to the extent 10-30% in non dose dependent manner.

B. *In vivo* studies :

(i) LD₅₀ : The highest dose of fraction (F-X) (1600 mg/kg) did not kill any of the animals in the group upto 48 hours, hence LD₅₀ could not be calculated.

(ii) The antiinflammatory, analgesic and anticonvulsant studies of fraction (F-X) did not reveal any significant activity, worth mention.

Discussion :

The present study indicates that fraction (F-X), a semipure mixture of chemically related compounds, from the bark *B. monosperma*, possesses both bronchodilator as well as antianaphylactic activity.

The fraction showed bronchodilator activity, as evidenced by results of *in*

vitro and *in vivo* tracheal smooth muscle experiment. The effect is mediated through beta adrenergic receptors as propranolol caused a blockade of fraction (F-X) responses on these preparations.

The fraction demonstrated a powerful anti-anaphylactic activity. Though an impure compound, fraction (F-X) is more active than DSCG locally, while isoprenaline is 10 times more active than the fraction. By i.p. route DSCG is almost 100 times more active than fraction (F-X). The higher dose required by i.p. route may be due to inadequate absorption of fraction (F-X). This action is also mediated through beta-adrenergic receptor system, since propranolol block the inhibitory action of fraction in both models.

The fraction also exhibited a weak cardio-stimulant activity in the isolated guinea pig atria and a non-specific partial blockade of standard agonists by fraction (F-X) in isolated guinea pig ileum and rat uterus supports its smooth muscle relaxant activity.

Thus fraction (F-X) demonstrates an anti-anaphylactic activity. The fraction also possesses mild cardio-stimulant activity. Apart from these the fraction does not possess any other effect. In the view of its potent antianaphylactic activity, especially since the fraction is not a pure compound, the fraction merits further study.

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Studies on an Autocoid Liberator from *Dioscorea hispida*

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The non-polar fraction of *D. hispida* has been investigated for its effect on autocoid release. Studies on oedema response in mice paw, dye extravasation in rat skin and spasmogen release from platelets, using a specific autocoid antagonist, revealed that the fraction induces a release of 5 HT. However the fraction did not cause aggregation of platelets thus suggesting the involvement of a specific release process rather than non-specific cellular damage.

Introduction

A wide variety of substances both of natural and synthetic origin have been demonstrated to cause a release of bio-active substances from their depots. Important among these are hyaluronidase (Elster *et al.* 1949), compound 48/80 (Paton 1951) and dextran (Halpern *et al* 1954). The mediators released by such agents are histamine (Halpern and Briot 1952), 5-hydroxytryptamine (5-HT) (Halpern *et al* 1957), SRSA (Chakravarty *et al* 1959) and possibly anaphylotoxin (Halpern *et al* 1954).

While investigating the non-polar fractions of *D. hispida*, it was observed that, their effects could be due to release of an endogenous autocoid, *D. hispida* is a climber, the tubers of which are extremely toxic and have been used as poisons for hunting. While the neuromuscular blocking action of the tubers, no doubt contributes significantly towards its toxic manifestations, reports of human poisoning describe symptoms such as laryngeal oedema which could be attributed to mediator release. In addition several dermal reactions were

noted in our laboratory while handling the fresh tubers.

A possibility that some fractions of *D. hispida* could cause specific mediator release thus existed. The present report details the investigations carried out to study mediator release by non-polar fractions of *D. hispida*.

Methods :

Phytochemical : Fresh tubers of *D. hispida* were cut into pieces and were extracted by ethanol at room temperature. The ethanol extract was acidified to PH 3.5 using citric acid. Hexane extraction was carried out so as to obtain the non-polar constituents.

The hexane extract was chromatographed by TLC using chloroform : methanol 9 : 1 to isolate the non-polar constituents (F 1).

Pharmacological : (A) Mediator release was studied using the method of Ghooi *et al* (1980, 1981) on the mouse hind foot pad. On this model the following experiments were carried out. (1) Dose response curve for oedema formation by F1: 48/80 and 5-HT. (2) Effect of speci-

fic antagonists of histamine and 5-HT.
(3) Effect of repeated daily doses of F1, 5-HT, and 48/80.

(B) Fraction F1 was studied for its effect on blood platelets *in vitro* with respect to 5-HT release and aggregation (Cusak 1980).

(C) Extravasation of the dye induced by F1 into skin of rats was measured by using the method of Martin 1971.

Results :

Phytochemical : The hexane extract of acidified ethanol extract on TLC demonstrated 8 fractions by iodine staining. The fraction corresponding to Rf values 95, 90, 82.5 were collected from preparative plates using chloroform as solvent. Of which fraction one i.e. F1 with Rf 95, amounted to 10.5% by weight of the total non-polar fraction. F1 was a yellow waxy compound which showed single spot on different TLC solvent systems such as Benzene : Methanol 9 : 1 and Chloroform : methanol 9 : 1.

Pharmacology : (A) (1) The oedema produced by F1, 5 HT and 48/80 was dose related as shown in Table 1.

(2) Methysergide bimaleate at a dose of 1 mg/kg intraperitoneally 30 min., before treatment with F1 produced a 70% inhibition of oedema, while mepyramine maleate at the same dose was without effect. Results of antagonists on oedema induced by F1 and standard agent are shown in Table 2 and 3.

(3) F1 on repeated doses produced tachyphylaxis with respect to oedema genesis. 5-HT however showed no tachyphylactic responses and its response in animal pretreated with F1 was not altered (see Table 4).

(B) The fraction F1, caused no aggregation of platelets in doses ranging from 10 mcg/ml to 100 mcg/ml. At a dose level of 2 mcg/ml the fraction caused the liberation of spasmogen, which was identified as 5 HT by assay of rat fundus and inhibition by methysergide bimaleate at 1 mcg/ml. See Fig. 1.

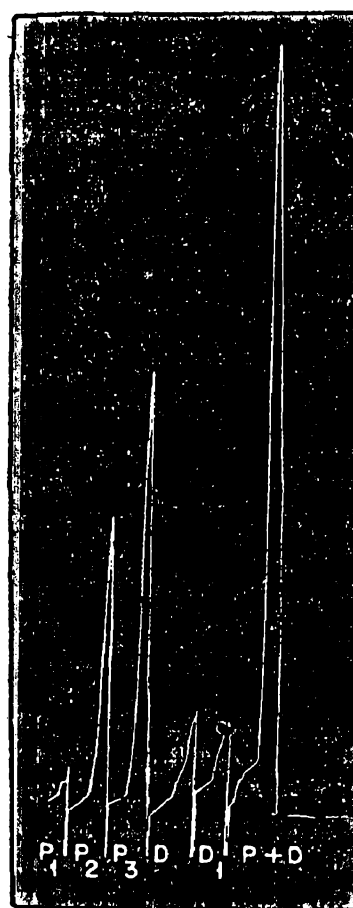


Fig. 1,

Effect of plasma on isolated rat fundus strip.

P1, P2, P3 = 0.1, 0.5, 1.0 ml. platelet rich plasma.

D, D1, = 5 and 2 mcg/ml.

P + D = 0.5 ml. platelet rich plasma and 2 mcg of F1 were incubated at 37°C for 10 minutes.

TABLE — 1
Induction of mouse paw oedema by
different agents

Agent	Dose mcg/paw	Oedema Units	±S.E.
5-HT	0.1	16.2	1.11
	0.2	22.6	0.92
	0.4	32.4	0.92
F1	2.5	12.6	1.53
	5.0	17.0	2.12
	10.0	21.67	0.61
48/80	0.078	8.17	0.70
	0.156	17.6	0.80
	0.312	23.5	1.05

TABLE — 2
Effect of 5 HT antagonist on paw Oedema

Treatment	5 HT 0.2 mcg Oedema Units	±S.E.	F1 10 mg. Oedema Units	±S.E.
—	22.6	0.92	21.0	1.44
Methysergide	5.8	0.66	6.4	0.99

$P > 0.005$

$P > 0.005$

TABLE — 3
Effect of Mepyramine on F1, 48/80 and Polymixin B Induced Oedema

Agent (Dose)	Control Oedema Units	±S.E.	Mepyramine 1mg/kg i.p. Oedema Units	±S.E.
48/80 (0.3 μ g)	20.8	1.2	11.7	2.12*
Polymixin (4 μ g)	19.5	0.57	0.6	1.29**
P1 (10 μ g)	18.8	0.967	16.6	2.02***

* $P > 0.01$

** $P > 0.001$

*** $P > 0.1$

TABLE — 4
Effects of repeated dailydoses of F1 and 5 HT

Group	Day 1			Day 2			Day 3		
	Oedema	±S.E.	P	Oedema	±S.E.	P	Oedema	±S.E.	P
I	19.0	2.3	—	18.1	2.9	0.1	16.8	1.77	0.1
II	20.8	1.84	—	9.6	1.0	0.001	1.8	0.2	0.001
III	21.8	0.59	—	12.0	1.3	0.001	5.83	1.08	0.001
IV	20.6	0.95	—	5.5	2.2	0.001	16.8	1.12	0.05

Gr. I 5 HT 0.2 µg daily for 3 days.

Gr. II F1 10 µg daily for 3 days.

Gr. III 48/80 0.3 µg daily for 3 days.

Gr. IV F1 10 µg for 2 days followed by 5 HT 0.2 µg on 3rd day.

TABLE — 5
Skin extravasation of Evans blue (25 mg./kg. i.v.)

Agent	Dose ug/I.D.	Dye extravasation ug.	S.E.	% Inhibition
Saline	0.05ml.	1.515	0.240	
5 HT	10.0	29.4	0.93	
F1	500.0	11.7*	0.53	
5 HT + MS	10.0	2.216*	0.62	92.46
F1 + MS	500.0	3.35*	0.472	71.36

*P > 0.001

MS, Methysergide bimalate 1 mg/kg i.p.

(C) F1. on intradermal administration induced extravasation of Evans blue in the skin of rats. The Evans blue was administered intravenously to rats at dose of 25 mg/kg. The extravasation induced was inhibited by methysergide bimalate. (Table 5).

Discussion :

Plants and plant products exert a variety of effects on man and animals. While some constituents of the plant possess *per se* biological effects and might mimic autocoids, a large variety

exert their biological effects due to the release of endogenous substances. Autocoids release by synthetic and natural agents known is usually non-specific involving the simultaneous release of a number of performed mediators. (Chakravarty *et al* 1959).

The non-polar fraction of *D. hispida* exerts its effects by causing a specific release of 5 HT, from mast cells and platelets. Such a release has been previously demonstrated with compound 48/80 (Chasin *et al* 1979) and agents

like ADP, trypsin, thrombin (Moncada and Vane 1979). In contrast to the above mentioned compounds, F1 induced 5-HT release is not associated with concomitant release of other autocooids. Evidence for this is the absence of any effect of mepyramine on F1 induced oedema. Tachyphylaxis to the oedema induced by F1 gives direct evidence to the mechanism of action of the fraction.

Studies on dye extravasation in the skin at the site of injections further suggest that F1 causes extravasation due to release of 5-HT. Studies on platelet rich plasma further corroborate with the contention that F1 releases 5-HT. As stated earlier trypsin, thrombin and ADP

also cause the release of 5-HT from platelets, however this release is associated with aggregation. Studies on platelet aggregation suggest that F1 is without any such effect.

Thus 5-HT release by F1 could be a specific reaction not involving total degranulation or disruption/aggregation. For this reason the effect of F1 on autocooid stores needs further study to elucidate the exact mechanism of action.

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Inhibition of certain biological effects of diphtheria toxin by an indigenous agent : a preliminary report

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Diphtheria antitoxin is the only agent known to be useful *in vivo* for inhibiting the activity of diphtheria toxin. An indigenous agent that inhibits the inflammatory response to diphtheria toxin, both in the rabbit skin and in the mouse footpad, is demonstrated here. This raises interesting possibilities for therapeutics as well as for studying cell-toxin interactions.

Introduction :

So far, diphtheria antitoxin is the only agent available to prevent the biological effects of diphtheria toxin (1). However, there are quite a few claims from indigenous Indian systems of medicine regarding herbal or mineral remedies which have an antidiphtheritic effect (2). A few of these remedies were evaluated using their inhibition of diphtheria toxin effect as indication of activity. The model systems used were the intracutaneous injection of toxin in rabbits leading to induration which is used in a slightly different form for evaluating antitoxin potency by pharmacopoeial requirements (3), and a method using footpad oedema production in mice by the toxin, standardized in our laboratory. An indigenous agent that shows such inhibitory activity is reported. The drug preparation 'Gandhaka Dhruti' was prepared from colloidal sulfur and linseed oil using traditional methods of trituration, ripening and filtration.

Materials

Diphtheria toxin and antitoxin were obtained from Haffkine Biopharmaceutical

Corporation Ltd., Bombay-400 012. Diphtheria toxin, non glycerinated : 90 Lf/ml. Diphtheria antitoxin : 110 IU/ml.

The drug preparation was kindly supplied by Vaidya N. N. Bagewadikar of Solapur.

Male New Zealand rabbits weighing 1.5 — 2.0 kg, and male NIH mice weighing 18-22 gm were used.

Methods :

A. Dose-dependent induration produced by diphtheria toxin in rabbits :

Two rabbits were taken, and their backs depilated with a 2:1 mixture of starch and barium sulphide. Twentyfour hours later, 0.1 ml of diphtheria toxin in concentrations of 3.5 Lf/ml, 0.35 Lf/ml, 0.035 Lf/ml and 0.0035 Lf/ml respectively was injected intradermally at 2 sites on the backs of each rabbit. 0.1 ml of sterile normal saline was injected as control on each rabbit. The diameter of induration produced was measured at 48 and 72 hours. On the basis of these dose-response values, a dose suitable for the next experiment was selected.

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B. Inhibition of diphtheria toxin activity in rabbits :

Two groups of rabbits with 3 rabbits in each group were taken. One group was fed the drug at a dose of 2ml/kg daily by stomach tube for 3 days before challenge. The control group was fed saline. The backs of rabbits were depilated as before. Twentyfour hours later, 0.1 ml of toxin with a concentration of 0.35 Lf/ml was injected intradermally at each site at 2 points on the left side of the midline on the back. In the control group, on the right side on corresponding sites 0.1 ml of a toxin — antitoxin mixture was injected. The mixture had a toxin content of 0.35 Lf/ml and an antitoxin content of 0.35 IU/ml. Average diameters of the resulting induration were recorded 48 and 72 hours later. The animals were weighed before and after the experiment.

C. Oedema induced by diphtheria toxin in the mouse footpad :

Four groups of 6 mice each were taken. Dilutions of diphtheria toxin in normal saline in doses of 45, 30, 22.5 and 18 Lf/ml were used for the four groups respectively. The normal thickness of the hind footpads were recorded with a thickness gauge (Mitutoyo, Japan). Then, 0.05 ml of the appropriate toxin dilution was injected subcutaneously in the left hind footpad and 0.05 ml of normal saline in the right hind footpad. Twentyfour and 48 hours later the thickness of the footpads was measured with the gauge and the difference between the increase in the thickness of the left and right footpads was taken as a measure of oedema produced by the toxin.

D. Inhibition of the toxin activity in the mouse footpad :

Three groups of six mice each were taken. One group was fed the drug at a dose of 10 ml/kg for 3 days prior to challenge with the toxin. A control group was fed saline. A toxin concentration of 45 Lf/ml was used. Normal footpad thickness was measured and toxin was injected as described above in these two groups. In the third group 0.05 ml of a toxin-antitoxin mixture was injected in the left hind footpad. The mixture contained 45 Lf of toxin and 45 IU of antitoxin/ml. On the right side 45 IU/ml antitoxin alone in a dose of 0.05 ml was injected. Footpad thickness was measured in all groups at 24 hours and the differences in the increase of thickness between the left and the right pads was taken as a measure of oedema due to the toxin. Animals were weighed before and after the experiment.

E. Effect on carrageenan induced oedema in the mouse footpad :

To examine the possibility of the agent having some anti-inflammatory activity, its effect on oedema induced in the mouse footpad by carrageenan was studied.

Three groups of 6 mice each were taken. One group was fed the drug according to the schedule mentioned above. Another group was fed saline. The third group was treated with oxyphenbutazone 300 mg/kg I.P. 1 hour before challenge. Normal footpad thickness was measured and 0.05 ml of 1% carrageenan in saline was injected subcutaneously in the left hind footpad of all 3 groups and 0.05 ml of saline on the

right side. Footpad thickness was measured 2 hours later. The difference in increase in the footpad thickness between the two sides was taken as a measure of oedema due to carrageenan. Animals were weighed before and after the experiment.

Statistical analyses were done by Student's 't' test.

Table 1
Amount of induration produced by diphtheria toxin
Induration diameter in mm.

Dose	48 hours	72 hours
3.5 Lf/ml	Necrosis present	Massive sloughing started
0.35 Lf/ml	38.5	40.5
0.035 Lf/ml	24.0	25.5
0.0035 Lf/ml	18.0	19.0

Note: Figures indicate average readings.

Results:

A. Induration produced by diphtheria toxin in rabbits:

The average diameters of induration produced at the end of 48 and 72 hours are shown in Table 1.

The sites at which 3.5 Lf/ml of toxin was injected showed massive necrosis and sloughing of the skin at the end of 72 hours.

There was a dose-dependent induration produced by the toxin. For the inhibition experiment a toxin dose of 0.35 Lf/ml was chosen.

B. Inhibition of diphtheria toxin activity in rabbits:

Rabbit skin induration was significantly less in the drug treated group as compared to control group by about 25% at 48 hours and 15% at 72 hours (see table 2). Two rabbits out of the 3 control animals died on the 6th day. There were no deaths in the treated group.

Table 2
Effect of the drug on rabbit skin induration due to diphtheria toxin

Group	Induration diameters in mm.	
	48 hours (Mean $\pm$ S.D.)	72 hours (Mean $\pm$ S.D.)
Control (6)	36.5 $\pm$ 4.13	39.5 $\pm$ 4.42
Toxin + antitoxin (6)	3.67 $\pm$ 1.208*	4.16 $\pm$ 0.7523*
Drug (6)	28.5 $\pm$ 5.51*	33.5 $\pm$ 2.26*

Note: The figures in brackets indicate the number of observations.

* $P < 0.05$

Table 3

Oedema in the mouse footpad with graded doses of diphtheria toxin over 72 hours

Dose	Oedema in the footpad (1 unit = 0.001 inch)		
	24 hours (Mean $\pm$ S.D.)	48 hours (Mean $\pm$ S.D.)	72 hours (Mean $\pm$ S.D.)
45 Lf/ml (6)	19.0 $\pm$ 0.89	11.5 $\pm$ 1.87	11.0 $\pm$ 2.0
30 Lf/ml (6)	15.33 $\pm$ 0.52	7.5 $\pm$ 1.05	7.33 $\pm$ 2.34
22.5 Lf/ml (6)	12.33 $\pm$ 0.68	8.0 $\pm$ 2.0	6.8 $\pm$ 1.96
18 Lf/ml (6)	9.66 $\pm$ 1.63	7.5 $\pm$ 1.51	6.0 $\pm$ 1.1

Note: Figures in brackets indicate number of observations.

C. Oedema induced by diphtheria toxin in the mouse footpad:

Mouse footpad oedema due to diphtheria toxin was dose dependent over a range of 45 Lf/ml to 18 Lf/ml. The peak oedema was observed at 24 hours (see table 3).

Therefore a dose of 45 Lf/ml was chosen to study inhibition by the drug and the 24 hour reading was taken as the representative response.

D. Inhibition of the toxin activity in the mouse footpad:

When the above dose was used in control and drug-fed groups, the drug fed group showed a significant decrease in oedema at 24 hours by about 30%. The group which was challenged with a mixture of the toxin and antitoxin showed a significant decrease in oedema by about 70% (See table 4).

Table 4

Inhibition in the footpad oedema after treatment with the drug

Group	Oedema at 24 hours (1 unit = 0.001 inch) (Mean $\pm$ S.D.)
Control (6)	22.16 $\pm$ 2.07
Toxin + antitoxin (6)	7.2 $\pm$ 3.06**
Drug (6)	15.33 $\pm$ 2.15**

Note: Figures in brackets indicate number of the observations.

**P < 0.001

E. Effect of the agent on carrageenan-induced oedema in the mouse footpad:

Carrageenan induced oedema in the mouse footpad was significantly less by about 40% in the group treated with oxyphenbutazone. However, pretreatment with the drug did not have any effect on the carrageenan-induced oedema (See Table 5).

Table 5
Effect on oedema produced by injection of carrageenan in the mouse footpad

Oedema at the end of 2 hours (1 unit = 0.001 inch)	
Group	(Mean $\pm$ S.D.)
Control (6)	25.66 $\pm$ 3.83
Oxyphenbutazone (6)	16.5 $\pm$ 2.26**
Drug (6)	26.5 $\pm$ 4.23

Note: Figures in the brackets indicate number of observations.

** P < 0.001

There were no differences in the weights of animals during the experiments.

Discussion :

Diphtheria toxin exerts its biological activity by binding to specific receptors on mammalian cells and entering the cytoplasm. In the cytoplasm it inactivates elongation factor -2 (EF-2), a crucial factor responsible for polypeptide synthesis (4). It thus brings about inhibition of protein synthesis, and, after a lag period, cell death. There are no agents known which inhibit the cytoplasmic action of diphtheria toxin *in vivo*,

though agents like nicotinamide or even an increase in pH are known to reverse the inactivation of the elongation factor *in vitro* (4). However, the concentration of nicotinamide or H⁺ needed is toxic to cells (4). For inhibition of the toxin receptor interaction, the sole therapeutically used agent is diphtheria antitoxin, though certain oligosaccharide residues are also known to inhibit the interaction (5). An indigenous agent that protects against certain biological effects of diphtheria toxin *in vivo* has been reported here. The agent does not possess any antiinflammatory activity. Elucidation of the mechanism of action would be of interest not only for its therapeutic implications but also for the light it may shed upon cell-toxin interactions.

Acknowledgements :

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***Semecarpus anacardium*. Separation of the Bhilawanols
A and B and a comparative study of their growth
inhibitory effect on *Clostridium tetani*
and general pharmacology**

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The closely related monoene and diene bhilawanols — bhilawanol A 1/bhilawanol B 2 — have been isolated pure by hydrolysis of their diacetates 3/4 separated by Ag⁺ ion chromatography of the acetylated extract of the nuts of *Semecarpus anacardium*. A comparative study of the growth inhibitory effect of the two bhilawanols on *Clostridium tetani* has revealed significant activity for both, the diene 2 being twice as potent as the monoene 1.

Introduction

The Marking Nut, *Semecarpus anacardium* Linn (Hindi : bhilawa ; Marathi : biba. Family : Anacardiaceae) is widely used in Ayurvedic medicine for a variety of ailments¹. It has been reported to possess, *inter alia*, antitumor and anti-infective properties. Patwardhan⁴ et al have recently reported significant growth inhibitory effect *in vitro* for the chloroform extract (essentially a mixture of bhilawanols A and B) towards *Clostridium tetani* and have also shown it to have no effect on a variety of aerobes tested. This paper deals with the isolation of the two pure bhilawanols A1 and B2 and a comparative study of their pharmacology and growth inhibitory effect on *Cl. tetani*.

Materials and Methods

Material : Freshly collected mature biba nuts were supplied by the Divisional

Forest Officer, Aurangabad, in March 1981.

Instruments : IR spectra were recorded on a Perkin-Elmer Infracord model 137E, PMR spectra were taken on a Varian T-60 spectrometer using TMS as internal standard. Mass spectra (MS) were obtained with a CEC spectrometer, model 21-110 B using an ionising voltage of 70 eV and a direct inlet system. Optical density was taken at 640 nm on a Bausch and Lomb Spectronic 20 colorimeter.

Experimental animals : All from Biopharmaceutical Corporation Ltd. All drugs and chemicals were of analytical grade and standard pharmacological equipment was used.

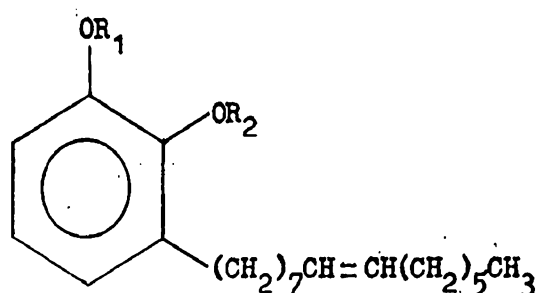
Inoculum used : 24 hour-old culture of *Cl. tetani* (N. S. Harvard adjusted to 50 per cent transmission at 640 nm.)

Media : Thioplycollate broth (Difco).

Extraction and acetylation.

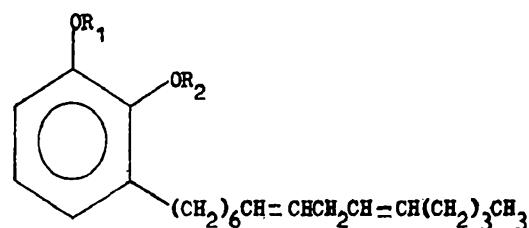
The crushed biba nuts (1Kg) were allowed to soak in CHCl_3 (3.5 litres) with frequent swirling at room temp. for 7 days when the extraction was complete. After complete removal of the solvent on a water bath under reduced pressure, a dark brown thick liquid (360 g) was obtained. Distillation of the crude extract (200 g) yielded the bhilawanols mixture as a yellowish liquid, b.p. $220^\circ/0.5$ mm (105 g). The distillate (70 g) in dry pyridine (70 ml), cooled in ice water, was treated with acetic anhydride (70 ml). After 18 hr. at ambient temp, the reaction mixture was diluted with water (200 ml), acidified with conc. HCl (congo red), extracted with benzene (3×100 ml), successsively washed with aq. NaHCO_3 , water, brine and dried. After removal of solvent the residue was distilled to furnish the bhilawanol diacetate mixture b.p. $215^\circ/0.5$ mm (65 g).

Separation of the acetylated bhilawanols mixture : Bhilawanol A-diacetate **3** and bhilawanol B-diacetate **4**. The acetylated mixture of bhilawanols (20 g) was chromatographed over 15% AgNO_3 - SiO_2 gel (500 g ; 95 cm x 4 cm) with Ag^+ - TLC monitoring of the fractions :



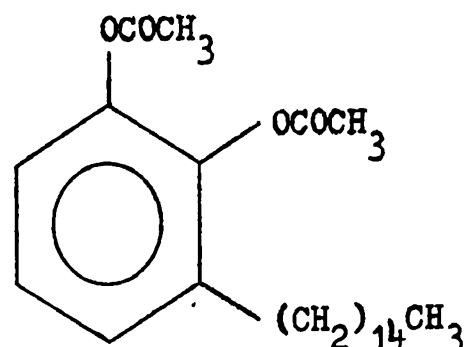
1: $\text{R}_1 = \text{R}_2 = \text{H}$

3: $\text{R}_1 = \text{R}_2 = \text{COCH}_3$



2: $\text{R}_1 = \text{R}_2 = \text{H}$

4: $\text{R}_1 = \text{R}_2 = \text{COCH}_3$



5

Fr 1 light petroleum - C_6H_6 (1:1) 3×200 ml, 1.0 g. saturated bhilawanol diacetate - **5**

Fr 2 light petroleum - C_6H_6
 7×200 ml, 6.0 g, bhilawanol A - diacetate, pure - **3**

Fr 3 light petroleum - C_6H_6
 10×200 ml, 5.0 g, Mixture **3** + **4**

Fr 4 C_6H_6
 7×200 ml, 7.0 g, bhilawanol B - diacetate, pure **4**

Bhilawanol A-diacetate 3

Distillation of Fr 2 gave **3** as a yellowish liquid, b.p. 245° (bath)/0.5 mm. IR (smear) : 1750, 1610, 1590 cm^{-1} . PMR (CCl_4) : 7.03 ppm (essentially s, 3H, aromatic H), 5.27 ppm (t, 2H, olefinic

H, $J=4\text{Hz}$), 2.50 ppm (t, 2H, Ar - CH_2 , $J=8\text{Hz}$), 2.27 and 2.20 ppm (two s, 3H each, $\text{OCOCH}_3 \times 2$), 0.90 ppm (fully distorted t, 3H, $(\text{CH}_2)_n \text{CH}_3$). MS: m/e 402 (M^+). (Found: C, 75.35; H, 9.59. $\text{C}_{25}\text{H}_{38}\text{O}_4$ requires: C, 74.59; H, 9.52%).

Bhilawanol B- diacetate 4

Fr 4 was distilled to obtain 4 as a yellowish liquid b.p. 250° (bath)/0.5 mm: IR (smear): 1750, 1600, 1580, 1220 cm^{-1} . PMR (CCl_4): 7.10 ppm (essentially s, 3H, aromatic H), 5.33 ppm (t, 4H, olefinic H, $J=4\text{Hz}$), 2.73 ppm (t, 2H, $\text{CH}=\text{CHCH}_2$, $J=4\text{Hz}$), 2.50 ppm, 2.50 ppm (t, 2H, Ar- CH_2 , $J=8\text{Hz}$), 2.27 and 2.20 ppm (two s, 3H each, $\text{OCOCH}_3 \times 2$), 0.93 ppm (slightly distorted t, 3H, CH_2CH_3 , $J=6\text{Hz}$). MS: m/e 400 (M^+). (Found: C, 75.46; H, 9.18. $\text{C}_{25}\text{H}_{36}\text{O}_4$ requires: C, 74.96; H, 9.06%).

Hydrolysis of bhilawanol A-diacetate 3: Bhilawanol A 1

Bhilawanol A-diacetate 3 (5 g) in EtOH (100 ml) was cooled in ice water and treated with a cold 1% aq. ethanolic soln of KOH (100 ml). After 18 hr. at room temp. the mixture was diluted with water (150 ml), extracted with benzene (3 x 100 ml), washed successively with water, brine and dried. Removal of solvent followed by distillation of the residue in a bulb tube at 0.5 mm (free flame) yielded bhilawanol as a yellowish thick liquid (2.2 g). IR (smear): 3200, 16100, 1580, 1280, 1180 cm^{-1} . PMR (CCl_4): 6.57 ppm (s, 3H, aromatic H), 5.33 ppm (t, 2H, olefinic H, $J=4\text{Hz}$, superimposed on a broad multiplet due to OH x2), 2.70 ppm (t, 2H, $\text{CH}=\text{CHCH}_2$, $J=4\text{Hz}$), 2.53 ppm (t, 2H, Ar-

CH_2 , $J=8\text{Hz}$), 0.90 ppm (fully distorted t, 3H, $(\text{CH}_2)_n \text{CH}_3$). MS: m/e 318 (M^+). (Found: C, 77.92; H, 10.90. $\text{C}_{21}\text{H}_{34}\text{O}_2$ requires: C, 79.19. H, 10.76%).

Hydrolysis of bhilawanol B-diacetate 4: Bhilawanol B 2

Bhilawanol B-diacetate 4 (6 g) was hydrolysed with 1% aq. alcoholic KOH (110 ml) and the product isolated as described above to yield bhilawanol B 2 as a yellowish liquid (2.6 g). IR (smear): 3200, 1605, 1570, 1275, 1180 cm^{-1} . PMR (CCl_4): 6.57 ppm (s, 3H, aromatic H), 5.30 ppm (t, 4H, olefinic H, $J=4\text{Hz}$; superimposed on a broad multiplet due to OH x 2), 2.70 ppm (t, 2H, $\text{CH}=\text{CHCH}_2$, $J=4\text{Hz}$), 2.53 ppm (t, 2H, Ar- CH_2 , $J=8\text{Hz}$), 0.90 ppm (slightly distorted t, 3H, CH_2CH_3 , $J=6\text{Hz}$). MS: m/e 316 (M^+). (Found: C, 78.10; H, 10.23. $\text{C}_{21}\text{H}_{32}\text{O}_2$ requires:

Isolated guinea pig tracheal chain⁵ (Castillo & DeBeer 1947):

Isoprenaline was used as a reference drug to cause relaxation of the tracheal chain. Effect of bhilawanols A and B *per se* or on the submaximal isoprenaline (2 ng/ml) induced relaxation of the smooth muscle was then recorded in the dose range of 100 — 2000 mcg/ml.

Isolated guinea pig atria⁶ (Clark 1956):

Adrenaline was used as a standard drug which caused an increase in force and rate of the contractility of atria, dose dependently in the range of 8-32 ng/ml. Bhilawanols A and B were studied in doses ranging from 10 — 1000 mcg/ml for their effect on atria.

Isolated rat phrenic nerve diaphragm⁷
(Bulbring 1946):

The phrenic nerve was stimulated at voltage 2.4 V at 0.1 Hz and 0.2 m sec pulse width with a square wave stimulator. Bhilawanols A and B were studied at a dose range of 10 — 625 mcg/ml.

LD₅₀

LD₅₀ of bhilawanols A and B were carried out according to the method of Litchfield & Wilcoxon⁸. The bhilawanols 1 and 2 were injected intraperitoneally in mice in doses ranging from 62.5 to 500 mg/kg. Six animals were included in each group and the mortality upto 72 hours was noted; LD₅₀ was calculated from the graph.

Effects on *Cl. tetani*

The inhibitory effect of the bhilawanols 1 and 2 was studied by serial dilution method. Growth was measured as the change in optical density at 640 nm. For the determination of minimum inhibitory concentration (MIC), different concentrations of compounds 1, 2, 3 and 4 were prepared in sterile ethylene glycol, from 100 mcg/ml stock in a dose range of 0.078 to 20 mcg/ml. Medium used for assay procedure contained 4.5 ml sterile thioglycollate broth per tube. Inoculum (0.1 ml) was aseptically transferred to each of these tubes followed by the addition of 0.5 ml of the prepared concentrations of compounds 1, 2, 3, 4 in the respective sets. Tubes of positive, negative and ethylene glycol controls were also included in each set. Optical density was taken of each tube before incubation and after 24 hours anerobic incubation at 35°. Each set was carried out in triplicate and average of the O.D.

readings was taken for the determination of percent inhibition. 0.22 O.D. was considered as 100% growth after 24 hours incubation as evidenced by the positive control reading. Percent growth was calculated for each of the tubes from the respective sets by the above relation and was then converted to percent inhibition. The MIC was obtained as the minimum concentration of drug which showed 100% inhibition.

Results and Discussion

The reported biological studies on *S. anacardium* have been generally carried out on the chloroform extract of the nuts (essentially a mixture of the bhilawanols — A1 and B2). The desirability of having pure compounds for bioassay studies prompted us to isolate pure bhilawanol A and pure bhilawanol B from a mixture. The separation of the two closely related monoene 1/diene 2 bhilawanols has now been achieved by a combination of the silver nitrate impregnated silica gel chromatography of the acetylated mixture of bhilawanols followed by hydrolysis of the easily separated pure diacetates 3 and 4. The purity of the monoene/diene bhilawanol 1/2 was established by the correct integration of the olefinic protons (2H for 1 and 4H for 2). In addition, while the PMR spectra of the monoene bhilawanol 1 was characterized by only one triplet at 2.53 ppm (benzylic CH₂), the diene bhilawanol 2 displayed an additional downfield triplet at 2.70 ppm (doubly allylic methylene present only in 2).

Isoprenaline used as a reference standard caused a dose-dependent (2-8 ng/ml) relaxation of tracheal smooth muscle. Bhilawanol 1/2 did not produce

any *per se* relaxation and also failed to potentiate the submaximal dose (2 ng/ml of isoprenaline). Adrenaline was used as a standard drug on atria, it caused an increase in force and rate of the contractility of atria dose dependently in the range of 8-32 ng/ml. Bhilawanol 1/2 in the dose range of 1-1000 mcg/ml was found to have no *per se* effect on the preparation isolated phrenic nerve diaphragm. Bhilawanols 1 and 2 were studied at a dose range of 10-625 mcg/ml and found not to cause any change in the contraction of the diaphragmatic

muscle, induced by electrical stimuli indirectly through nerve stimuli or directly upto 15 minutes after addition of the drug. The LD₅₀ of bhilawanol 1 and 2 were found to be 154.9 and 85.11 mg/Kg respectively.

The minimum inhibitory concentration of 1 was found to be 2.5 mcg/ml and that of 2 was found to be 1.25 mcg/ml. These values are comparable with the MIC of the crude and distilled chloroform extract (crude : 5 mcg/ml ; 2.5 mcg/ml);

Table 1

Effect of Bhilawanol A 1 and Bhilawanol B 2 on the growth of *clostridium tetani*

Tube No.	Effective Conc. mcg/ml	Optical density		% Growth		% Inhibition	
		1	2	1	2	1	2
1	20	0.00	0.00	0.00	0.00	100	100
2	10	0.00	0.00	0.00	0.00	100	100
3	5	0.00	0.00	0.00	0.00	100	100
4	2.5	0.00	0.00	0.00	0.00	100	100
5	1.25	0.09	0.00	40.90	0.00	59.0	100
6	0.625	0.12	0.10	54.54	45.45	45.46	54.55
7	0.312	0.13	0.125	59.09	56.81	40.91	43.19
8	0.156	0.22	0.16	100.0	72.72	0.00	27.28
9	0.078	0.22	0.22	100.0	100	0.00	0.00
10	E. G. control	0.22		100			0.00
11	+ ve control	0.22		100			0.00
12	- ve control	0.00		0.00		100	

Key :

E. G. Control : 4.5 ml thioglycollate broth + 0.5 ml ethylene glycol + 0.1 ml inoculum.

+ve Control : 4.5 ml thioglycollate broth + 0.5 ml distilled water + 0.1 ml inoculum.

-ve Control : 4.5 ml thioglycollate broth + 0.5 ml distilled water.

From the above results it is clear that the MIC value went on decreasing during the purification. The diene **2** was found to be more potent than the monoene **1**, indicating the importance of the number and position of the double bond on inhibition. The acetates **3** and **4** were found to be inactive suggesting the necessity of hydroxyl groups for inhibition. Also, the inhibitory effect was found to be dose-dependent (See Table I). All aerobes tested were found to be resistant to both **1** and **2** even at higher concentrations (100 mcg/ml). The high susceptibility of aerobes of *clostridium* species is relevant for the specificity of the inhibitor.

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