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The chemistry and action of 6-alkylsalicylates of Indian *Ginkgo biloba*

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Five 6-alkyl (*n*-tridecyl-, *n*-pentadecyl-, *n*-heptadecyl-, *n*-pentadecenyl- and *n*-heptadecenyl-) salicylates **1a-e**, in free and conjugated (esters, phospholipids) forms, constituting one of the largest classes of bioactive metabolites have been isolated from the leaves and fruits of ten *Ginkgo biloba* trees of different ages (3-80 years) and indigenous occurrence. These compounds have been characterized by comprehensive chromatographic-spectroscopic analyses (HPTLC, HPLC, GC-MS, ^{1}H NMR), chemical transformation, and by direct comparison of some of them with authentic markers. Selective anti-oxidative (lipoxygenase modulatory), free radical captodative (^0OH, $^0SO_3^-$), and anti-allergic and anti-inflammatory screening of the total IGb extract and of **1a-e** have been conducted. Our findings suggest a significant contribution of the 6-alkylsalicylates towards the elixir effect of *Ginkgo*, contrary to an earlier apprehension that ginkgolic acids (equivalent to 6-alkylsalicylates) might cause allergic manifestations in recipients and, therefore, that they should be removed from *Ginkgo* formulations.

Herbalists fondly refer to *Ginkgo biloba* Linn. as the "living fossil", being the oldest living plant species with a 200 million-year history. It is the sole survivor of the family Ginkgoaceae[1]. *Ginkgo* was mentioned in the ancient Chinese pharmacopoeia for its many therapeutic uses[2]. Formulations of *Ginkgo* have, however, been introduced comparatively recently into the list of Occidental (Western) phytomedicines[3,4]. It is recommended in conditions related to geriatric complaints, peripheral circulatory insufficiency, and cerebrovascular disorders. The market of *Ginkgo* formulations in the Western countries had an estimated annual turnover of about 500 million US dollars in 1992-1993, which is steadily increasing[3,4]. But, surprisingly, no effort seems to have been made to explore the potential of Indian *Ginkgo biloba* (IGb) although a number of *Ginkgo* medicines from the West find a lucrative Indian market.

We have collected *G. biloba* (IGb) plant materials from different parts of India and established their identities pharmacognostically and chemically following established procedures (see Experimental Section). Phenolic lipids constitute one of the three major classes of secondary metabolites of *G. biloba* Linn., the other two being terpenoidal lactones and flavonoids. However, the former class of compounds was completely eliminated from the Western *Ginkgo* phytomedicines[4-6], due to the presumption that they cause allergic manifestations (of the urushiol-type) in recipients. We did not subscribe to this idea. We believe so long as the carboxyl group in 6-alkylsalicylates in intact (free or in conjugated form) there would not be any allergic reaction. Our findings reported herein validate this postulate.

Results and Discussion

(i)Isolation and structural study. Green leaves from ten *G. biloba* trees of widely ranging age-groups (3 to 80 years) were collected from different parts of India (during August-October, for two consecutive years) and processed for 6-alkylsalicylates (see Experimental). These phenolic lipids are represented by the general structures **1a-e** (Figure 1). The identities of these compounds were established by comprehensive chromatographic-spectroscopic analyses (HPTLC, HPLC, GC-MS. ^{1}H NMR), chemical transformations (selective deacylation, methylester acetate formation trimethylsilylation), and by direct comparison, where possible, with authentic markers.

The acetylmethyl esters exhibited characteristic proton resonances, in their ^{1}H NMR spectra, at δ0.85-0.88 (t, $-CH_3$), 1.25 (m, $-CH_2-$), 1.55(m, $Ar-CH_2-CH_2-$), 1.95 (m,

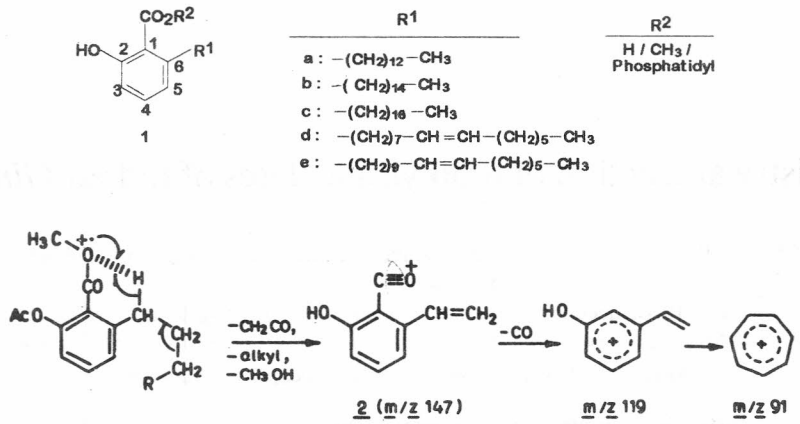


Figure 1—Structure and common mass fragment ions of 6-alkylsalicylates from *Gingko*

$-CH_2-CH=$), 2.20(s, CH_3CO), 2.70(t, $Ar-CH_2-$), 3.82(s, $-CO_2CH_3$), 5.3(m, $-CH=CH-$), 6.8-7.2 (m, $Ar-H$). In the EI MS, aside from the respective molecular-ion peak, characteristic common fragment-ion peaks appeared at m/z corresponding to M-42($-CH_2CO$) and M-74($-CH_3OH$, $-CH_2CO$). Additionally, prominent fragment-ion peaks appeared at m/z 147 (**2**), 119 (**2** $-CO$), and 91 (base peak arising from the tropilium ion, characteristic of the alkylaryl moiety) (Figure 1).

The relative abundances (by GC-MS, HPLC) of the 6-alkylsalicylates were reasonably consistent being in the ranges $15\pm5\%$ (**1a**), 7 ± 3 (**1b**), 4 ± 1 (**1c**), 65 ± 12 (**1d**), and 9 ± 3 (**1e**). They altogether comprised about 5% of the total lipids. Moderate concentrations of phospholipids (0.1-0.8% wet wt.) were also isolated, the range depending on the age of the tree. The lipids of immature fruits, on the other hand, were dominated but more variably upto $70\pm20\%$ by phenolic lipids, free and conjugated.

Aside from the complex lipoidal entities, two other major groups of compounds isolated from the different parts of the Indian *Ginkgo*, were flavonoids (free and glycosylated/conjugated) (yield, 0.2-0.5%) and terpenoidal lactones (ginkgolides and bilobalide, 0.002-0.08%). Some of the flavonoid compounds were strikingly found encaged within the cavity of the ginkgolide lactones (The significance of this aspect warrants further investigation as the reports made so far have not recorded this observation).

The 6-alkylated phenolic acids of *Ginkgo* were outrightly neglected earlier[4,6] probably because of their structural similarity to the C_{20}-C_{24}[(C_7+C_{13})-(C_7+C_{17})] fatty acids. However, unlike those of the long chain fatty acids (derived from poly-β-ketide intermediates), the biosynthesis of the side chain and of the salicylate ring of the 6-alkylsalicylates involve separate sequences, building blocks, subcellular sites and different levels of cell activation in *G. biloba*.[7]

(ii) Survival mechanism in *Ginkgo* Several genera of the family Ginkgoaceae occupied the Earth's ground surface some 200 to 100 million years ago[1]. Fossil *Ginkgos* were also found in the Indian sub-continent (we have access to one such precious *Ginkgo* fossil from Rajasthan), America, Australia and Europe. All the *Ginkgo* species, however, became extinct with the passage of time except *G. biloba*. What is the secret of its longevity?

G.biloba efficiently slows down the biochemical process of aging. It is able to reproduce from the age of about 20 years and can continue to do so more than 1000 years[1]. *Ginkgo* smoothly fends off several static and dynamic stresses (cosmic radiation, relentless atmospheric oxidation, microbial infestation, environmental pollution) and the adult respiratory syndromes (ARDS), presumably by employing its low and medium M_r metabolites that are produced in increased quantities in stress (e.g. pruning) situations. Oxygen free radicals are strongly implicated in the process of biologic aging in all living species[8,9]. Hence, in *G. biloba*, the antioxidative/radical captodative effects are manifested best. The flavonoid consituents of *Ginkgo* even though were reported[4,6,10] to attenuate the adverse manifestations of systemically generated superoxide radical ($O_2^{-\cdot}$), however, neither they nor the congeneric terpenoidal lactones exhibited[10] any antioxidative or radical captodative effect when challenged with the most damaging hydroxyl (OH) or sulphur-containing radicals.

(iii) Activity studies. We have now tested the

Table I—Effects of *Ginkgo* extract, metabolites and formulations on free radical induced polymerization of methyl methacrylate

Group[a]	Inhibitor (mg)	Induction period (min)[b]	Amount of polymer (mg)	(Polymer-type)
Control	—	5	648 ± 20	(PMMA-Fenton)
MMA + H·				
IGb + MMA + ·OH	20	(inf. > 300)	22 ± 5	(PMMA-IGb)
1a-e + MMA + ·OH	20	(inf. > 300)	38 ± 7	(PMMA-**1a-e**)
Ger + MMA + ·OH	40	30	602 ± 24	(PMMA-Gcr)
Rkn + MMA + ·OH	40	32	552 ± 28	(PMMA-Rkn)
Bvs + MMA + ·OH	40	28	588 ± 34	(PMMA-Bvs)
MMA + $\dot{S}O_3^-$	—	15	782 ± 14	(PMMA-SO_3^-)
IGb + MMA + $\dot{S}O_3^-$	20	150	112 ± 8	(PMMA-IGb-SO_3^-)
1a-e + MMA + $\dot{S}O_3^-$	20	122	94 ± 6	(PMMA-**1a-e**-SO_3^-)
Gcr + MMA + $\dot{S}O_3^-$	40	25	690 ± 24	(PMMA-Gcr-SO_3^-)
Rkn + MMA + $\dot{S}O_3^-$	40	25	705 ± 26	(PMMA-Rkn-SO_3^-)
Bvs + MMA + $\dot{S}O_3^-$	40	25	744 ± 28	(PMMA-Bvs-SO_3^-)

[a] Mean of ten replications

[b] Period between completion of addition of reactants and onset of precipitation, IGb, Indian *Ginkgo biloba* extract; Gcr, Ginkocer; Rkn, Rokan; Bvs, Bilovas; inf; infinite time > 300 min.

anti-hydroxyl and anti-sulphite anion radical ($\dot{S}O_3^-$) effects of the total leaf-extracts of IGb and of the 6-alkylsalicylates (**1a-e**) against polymerization of methyl methacrylate (MMA)[11]. Both these agents significantly protected MMA from polymerization by hydroxyl or sulphito-anion radicals. In contrast, some of the commercial *Ginkgo* products, e.g. Ginkocer, Bilovas and Rokan, prepared from a patented formulation (EGb-761)[5,6], were found to be totally ineffective in this parameter (Table I). The molecular participation of **1a-e** in the polymerization reaction was suggested from the dielectric properties (Table II) of the products (high dissipation factors of the PMMA polymers). The small amount of the polymers (PMMA) produced, with much shorter chain length than that of the control (without the inhibitors), also suggested that the phenolic ligands were incorporated into the PMMA polymers. This phenomenon had precedent in the MMA protecting effects of shilajit[11] and of mangiferin[12] under similar conditions.

The beneficient effects of IGb and **1a-e** associated with their anti-free radical properties were reflected in the pharmacological and immunological profiles of these agents. Literature reports[3-6] suggested that no biological screening data are available for the phenolic lipids of *Ginkgo*. In the present study, the effects of IGb and **1a-e** as anti-inflammatory and anti-allergic agents and on the biosynthesis of eicosanoids in Ca-ionophore activated human polymorphonuclear leukocytes (PMNL) were evaluated.

IGb (50 mg/kg, i.p.) and the 6-alkylsalicylates (**1a-e**, 50 mg/kg, i.p.) markedly attenuated carrageenan-induced pedal oedema in albino rats at 3 and 4 hr. The anti-inflammatory effect of these agents was not manifested at the initial stages (1 and 2 hr) of the inflammation, indicating that the effect is mediated by certain other effector molecules whose identity(ies) is not known at present. However, when IGb (50 μg/mL) and the 6-alkylsalicylates (50 μg) were administered intracerebroventricularly (i.c.v.) into the rats, there was significant inhibition of the pedal oedema throughout the 4hr period of observation.

Table II—Dielectric properties of polymethyl methacrylates with or without inhibitors[a]

Polymer	Dissipation factor (tan δ)[b]	
	β-relaxation	α-relaxation
PMMA-SO_3^-	0.0167	0.0169
PMMA-IGb-SO_3^-	0.0208	0.0240
PMMA-**1a-e**-SO_3^-	0.0210	0.0238
PMMA-Fenton	0.0239	0.0236
PMMA-IGb	0.0199	0.0316
PMMA-**1a-e**	0.0248	0.0333

[a] Dielectric properties were measured at frequency, 1 MHz; temp. 20°C.

[b] Mean of ten replicates (see Experimental Section).

Delayed-type hypersensitive reactions in the animal skin are often produced by foreign materials, e.g. dinitrofluorobenzene (DNFB) and picryl chloride, which are capable of binding to body constituents to form antigens. Contact dermatitis can occur in humans and in animals who become sensitive to such chemicals (potential antigens). IGb (as 2% cream) and 6-alkylsalicylates (0.2% cream) appreciably inhibited (30 ± 5%) the DNFB-in-

duced contact dermatitis in rat ear. These *Ginkgo* agents did not produce any allergic manifestation *per se*. The 6-alkylsalicylates produced a biphasic (dose-dependent) response on the biosynthesis of the leukotrienes (LTB_4 and $\omega-OH-LTB_4$) and the hydroxy-eicosatetraenoic acids (5-HETE and 12-HETE) suggesting its immuno-modulator (biological reponse modifier, BRM) effect on the lipoxygenase pathway(s).

It seems appropriate now to correlate, at the molecular level, the contributions of the phenolic lipids to the producer organism. Oxygen uptake by the effector proteins (oxygenases) of *G. biloba* is probably accompanied by a concomitant release of anionic ligands (heterotrophic), e.g. the phenolic lipids. The phenolic lipids in turn may reduce the heat released upon oxygen binding of the proteins (cf. dielectric properties, Table II) and thereby contribute to lower the overall enthalpy change of the oxygenation reaction, a key factor in the longevity of biological species. The abundant presence and the production of these antioxidative and radical captodative agents, e.g., the phenolic lipids, would seem to counter the effects of oxidative stress to which *G. biloba* is relentlessly exposed.

Conclusion

Attention has been drawn, in this communication, to the rewarding aspects of chemical and biological evaluation of *Ginkgo biloba* Linn., occurring in India. The 6-alkylsalicylates, occurring in free and conjugated forms in all parts of *Ginkgo* have been shown as yet another major class of its bioactive agents. The significance of these phenolic lipids (general structure, **1a-e,** Figure 1) in the health and longevity of *G.biloba* and, hence, in the organisms ingesting *Ginkgo* is highlighted. Anti-oxidative, free radical ($\cdot OH, \cdot SO_3^-$) captodative anti-inflammatory and anti-allergic effects of the total extracts of *G. biloba* leaves of Indian origin (IGb) and of the 6-alkylsalicylates are reported. Herein we dispel the western equivocal notion that the phenolic lipids (equivalent to ginkgolic acids) might cause allergic manifestations in recipients and should be removed from the *Ginkgo* formulation, e.g. EGb 761. The present study has demonstrated, for the first time that the carboxy group containing 6-alkylsalicylates contributes positively towards the elixir effect of *G.biloba.*

Experimental Section

Plant materials—Leaves and fruits were periodically collected, for two consecutive years, from ten *G. biloba* trees of different age groups (3-5, 10-15, 20-30 and 60-80 years old), located at Dehra Dun, Saharanpur, Manali and Ooty. The findings reported here pertain to the collection during August to October. The herbarium specimens are preserved in the library of Prof. S. Ghosal.

The following techniques were applied for the isolation and characterization of the chemical constituents of IGb.

Column chromatography. Adsorbant: silicic acid (Mallinck drodt, ~100 mesh), solvents of graded polarity, e.g., *n*-hexane, hexane-diethyl ether and ether were used as eluants.

HPTLC-CAMAG TLC evaluation assembly (CATS 3.16/Scanner II V 3.14) applying both flouresence and quenching modes of detection was used; *n*-hexane-ether-acetic acid (80:19:1) was used as the developer.

GC—MS. Hitachi M-4100 instrument; OV-1 (30M × 0.33 mm), operated with graded increase in temperature from 80° to 280°C; ionization voltage 20 eV.

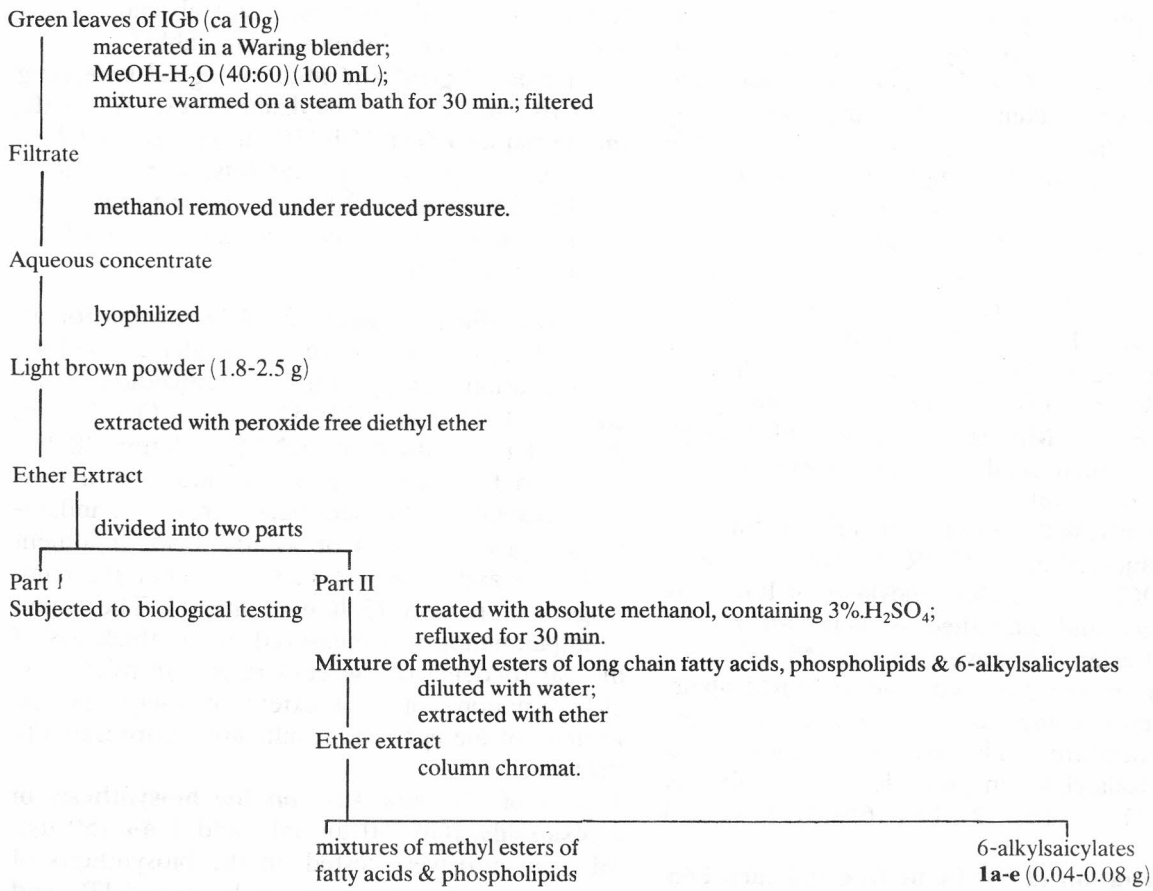
HPLC-Waters assembly; RP-8 reverse phase column equipped with a PDA detector: (i) methanol-water-acetic acid (80:20:0.2) [(ii) acetonitrile, as developers.

For comparison purposes, reference markers of anacardic acids (An 15:0 and An 15:1 as major entities) were employed in the HPTLC and HPLC analyses.

NMR-Instrument, Varion XL-100; solvent, $CDCl_3$. Chemical shifts (δ, ppm) were measured relative to tetramethylsilane (TMS) as internal standard.

Dielectric measurement-The dielectric properties of polymethyl methacrylates were measured using a Hewlett-Packard impedence analyzer, operating at 1 KHz to 1 MHz; the temperature was maintained at 20°C. The relaxation effects observed at 1 MHz are given in the Table II. The general method of isolation of the phenolic lipids is depicted in Scheme I.

Separation of 6-alkylsalicylates by column chromatography. The esters of fatty acids and the conjugated 6-alkylsalicylates were separated by column chromatography of the ether extract over silicic acid. The column was sequentially eluted with *n*-hexane (100%), hexane-diethyl ether (99.5:0.5, 99:1, 95:5, 90:10, 50:50) and ether (100). The eluants were combined according to TLC-HPTLC analyses of the aliquots. The hexane (100) and hexane-ether (99.5:0.5) eluates afforded a mixture of fatty esters and less polar phospho-



Scheme I-Isolation of phenolic lipids

lipids. The remaining eluates gave the 6-alkylsalicylates.

6-Alkylsalicylate-phospholipid conjugates. The combined *n*-hexane and hexane-ether (99.5:0.5) eluates was evaporated and the residue processed for phospholipids according to previously described procedures[13,14]. Briefly, the residue was extracted with pentane followed by aqueous methanol (20:80). The residue from the latter extract contained the phospholipid conjugates (38 mg).

The mixture of triglycerides, polyunsaturated fatty acids (PUFA), phenolic phospholipids (containing 6-alkylsalicylates at the C_2-position of glycerol moiety), and free 6-alkylsalicylates, obtained from the aqueous methanol extract of the leaves of *G. biloba* (IGb), were separated by column chromatography over silicic acid using eluants of graded polarity. The eluates were combined according to their TLC-HPTLC patterns. The *O*-acetylmethyl esters of 6-alkylsalicylates (**1a-e**) were prepared by acetylation (AC_2O-C_5H_5N) followed by methylation (Et_2O-CH_2N_2). These derivatives were separated by column chromatgraphy and also by semi-preparative reverse phase HPLC using methanol-water-acetic acid as developer. Deacylation of the phospholipids (22 mg) was carried out by treatment with a solution of anhydrous methanol-sodium methoxide (0.2 *N*, 0.5 mL) at room temperature for 1 hr. The solvent was removed and the residue extracted with pentane. The pentane-soluable fraction contained methyl esters of the fatty acids including PUFA. The pentane-insoluble residue was processed for phenolic entities in the usual way and the product extracted with ether. The residue from the ether extract was acetylated by treatment with acetic anhydride (0.5 mL) and pyridine (0.02 mL) at room temperature overnight. The acetate derivative, in chloroform, was passed through a short column of silica gel-H. Elution was carried out with pet. ether (40-60°C) and petrol-chloroform (99:1). The combined petrol-chloroform eluates on GC-MS analyses showed appropriate mass spectral patterns (Figure 1).

In another set, selective C_2-deacylation of the phospholipids was carried out by treating the residue from hexane eluates (column chromatogra-

phy) with phospholipase-A_2 (phosphatidyl-2-acylhydrolase, EC 3.1.1.4. Sigma Chemical Co.), at *p*H 8.9; 25°C for 12 hr. The liberated 6-alkylsalicylic acids were taken in ether and analysed by HPLC. The free 6-alkylsalicylic acids from IGb were isolated from the ether eluates of the column in a similar way. Thus, 6-alkylsalicylic acids were converted into *O*-acetylmethyl ester derivatives by treatment with AC_2O-C_5H_5N followed by methylation of the carboxyl group with ethereal diazomethane. TLC of the product in hexane-ether-acetic acid (74:25:1) showed acetylmethyl esters at R_f 0.64; UV: λ_{max} nm (log ε):240 (3.42), 290-92 (3.88) (in MeOH). The free 6-alkylsalicylic acids and their methyl esters showed R_f 0.45 and 0.35, respectively.

The *O*-acetylmethyl esters (general structure, **1a-e**) were subjected to ^1H NMR spectroscopic analysis in $CDCl_3$. The 6-alkylsalicylates of IGb were characterized and quantified as their O-trimethylsilylmethyl ester derivatives, by GC-MS. Silylation was done in pyridine with hexamethyldisilane-trimethylchlorosilane (2:1) according to a published procedure[15]. The average composition of the 6-alkylsalicylates in green leaves of IGb was, alkyl 13:0 (18%), 15:0 (2), 15:1 (65), 17:0 (5), 17:1 (10).

Polymerization of MMA by free radicals: Fenton reagent induced polymerization. IGb (20 mg) was dissolved in double distilled water (20 mL), through which N_2 was passed for 30 min. Ferrous sulphate (10 mg) and hydrogen peroxide (30%, 0.05 mL) were added, sequentially MMA (940 mg) was then added and the reaction mixture kept in a dark place, at ambient temperature (20 ± 5°C). The periods of onset and completion of polymerization were noted. The polymer (PMMA-IGb) was collected by filtration. The precipitate was repeatedly washed with water and dried *in vacuo*. The product was further purified by dissolving in benzene and re-precipitating with methanol. The weight of the pure, dry precipitate was noted (Table I). The polymerization of MMA in the presence of 6-alkylsalicylates to give PMMA-**1a-e**, and in the absence of any one of these inhibitors to give the control polymer (PMMA-Fenton), were carried out in a similar manner. Likewise, Ginkocer (PMMA-Gcr), Rokan (PMMA-Rkn) and Bilovas (PMMA-Bvs) were employed as potential inhibitors. The results are incorporated in Table I.

Sulphito anion radical induced polymerization of MMA. The SO_3^- radical was generated by interaction of potassium metabisulphite and ammonium persulphate as before[14]. The results of polymerization in the presence and absence of the above inhibitors are incrporated in Table I.

Pharmacological and immunological screening of IGb and the 6-alkylsalicylates: (i) Anti-inflammatory effect. IGb (50 mg/kg, i.p.) and **1a-e** (50 mg/kg, i.p.), in separate sets, were administered to albino rats (120-150g), prior to pedal inflammation induced by carrageenan (0.1 ml of 1% suspension in 0.9% saline)[16].

(ii)Anti-allergic effect. 2, 4-Dinitrofluorobenzene (DNFB) (0.5%), in ethyl alcohol-acetone (4:1) solution was applied for sensitization of the ear of albino rats (120-150g; b.w.). On the 5th day, after sensitization, DNFB solution (0.2%) was used for challenging and production of contact dermatitis in the sensitized zones. The inflammation produced with or without prior treatment with IGb and **1a-e** was measured after the challenge at 1 hr and 12 hourly interval. The extent of inflammation was measured by the thickness of the ear (oedema of the epidermis with microvesicle formation) and the extent of allergy by the redness of the ear. The results are incorporated in the text.

Effects of IGb and 1a-e on the biosynthesis of eicosanoids. IGb (50 μg/mL) and **1 a-e** (50 μg/mL), *in vitro* were tested on the biosynthesis of leukotrienes, viz. LTB and ω-hydroxy-LTB_4 and 5-hydroxyeicosatetraenoic acid (5-HETE) and 12-hydroxyeicosatetraenoic acid (12-HETE), in human PMNL, following a published procedure[17]. Human granulocytes were isolated by using dextran sedimentation, hypnotic lysis of erythrocytes and density gradient[18]. The granulocyte preparation contained 85-90% neutrophils (viability >95% as determined by trypan blue exclusion method). The identification of the eicosanoid products was done by HPLC (retention time, t_R) using authentic markers (Cascade Biochemical Ltd.).

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