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Antihepatotoxic effects of major diterpenoid constituents of *Andrographis paniculata*

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Abstract—The diterpenes andrographolide (I), andrographiside (II) and neoandrographolide (III) isolated from *Andrographis paniculata* were investigated for their protective effects on hepatotoxicity induced in mice by carbon tetrachloride or *tert*-butylhydroperoxide (tBHP) intoxication. Pretreatment of mice with the diterpenes (I, II & III; 100 mg/kg, i.p.) for 3 consecutive days produced significant reduction in malondialdehyde formation, reduced glutathione (GSH) depletion and enzymatic leakage of glutamic-pyruvate transaminase (GPT) and alkaline phosphatase (AP) in either group of the toxin-treated animals. A comparison with the known hepatoprotective agent silymarin revealed that I exhibited a lower protective potential than II and III, which were as effective as silymarin with respect to their effects on the formation of the degradation products of lipid peroxidation and release of GPT and AP in the serum. GSH status was returned to normal only by III. The greater protective activity of II and III could be due to their glucoside groups which may act as strong antioxidants.

Andrographis paniculata (Acanthaceae) known as Kalmegh in India is used as a bitter ingredient in the indigenous system of medicine. It is found in the plains of the West Indies, India and Sri Lanka. About 26 different polyherbal formulations of this plant are mentioned in Ayurveda as a popular remedy for the treatment of various liver disorders [1]. Kalmegh, which consists of the dried leaves and tender shoots of the plant, has aroused considerable interest for its beneficial effects in general debility, dysentery, dyspepsia, malaria, asthma, bronchitis, filariasis [2] and also in infective hepatitis during clinical studies [3].

Of the different diterpenes which have been isolated from *A. paniculata*, andrographolide (I*) is the major bicyclic diterpenoid lactone (Fig. 1) whose structure and configuration has been determined by a combination of NMR, MS, chemical transformations and X-ray crystallographic data [4, 5]. Previous studies have shown I to possess multiple pharmacological activities, such as, reduction in hexabarbital or phenobarbital sleeping time [6, 7], inhibition of drug metabolizing enzymes [8], antiperoxidative potential in the liver [9] besides choleretic and anticholestatic effects in animals [10]. These investigations have substantiated the therapeutic potential of I as a hepatoprotective agent. However, reports are not available in literature wherein the liver protective activity of *A. paniculata* has been attributed to other diterpene constituents i.e. andrographiside (II) [11] and neoandrographolide (III) [12] both of which are diterpene lactone glucosides (Fig. 1). Therefore, the present work was conducted to evaluate the hepatic efficacy of all three diterpenoids of *A. paniculata* in different liver damage models so that the contribution of each of these constituents in its hepatoprotective action could be determined.

Reduced glutathione (GSH) has been reported to serve as either a nucleophile forming conjugates with the active metabolites or as a reductant for peroxides and free radicals [13]. A decrease in GSH level may thus increase susceptibility of the tissue to oxidative damage like lipid peroxidation. In this study the effects of I, II and III on GSH status, malondialdehyde (MDA) formation and serum indices of hepatotoxicity produced by CCl_4 or *tert*-butylhydroperoxide (tBHP) intoxication in mice were investigated.

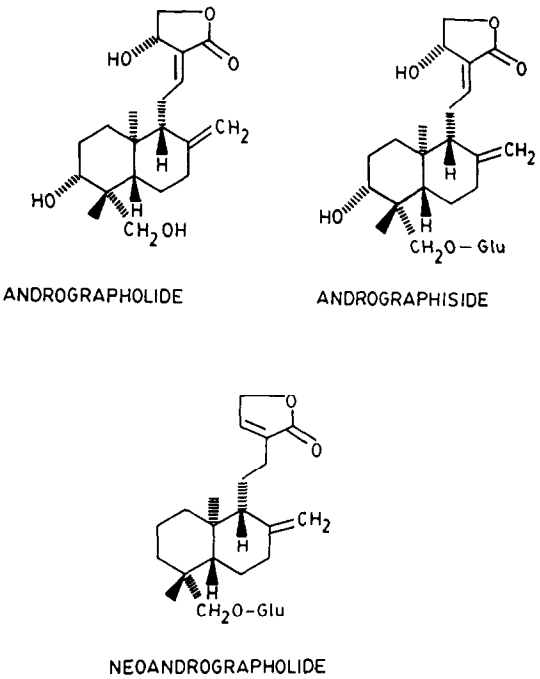

Fig. 1.

Materials and Methods

For isolation of the diterpenes the dried and powdered leaves of *A. paniculata* (4 kg) were extracted successively with chloroform and methanol in a soxhlet extractor. The chloroform extract, on concentration, deposited a solid which on crystallization from ethanol gave I as plates (20 g), m.p. 230–231°, $[\alpha]_D^{25°}$ – 124.4° (AcOH). The mother liquor from chloroform extract was evaporated to dryness and the residue chromatographed on a silica gel column. The chloroform eluate yielded a further crop of I (5 g). Elution with chloroform containing 2% methanol gave III as needles (from methanol; 1.7 g), m.p. 166–167°, $[\alpha]_D^{25°}$ – 47.1° (pyridine). The methanolic extract after

* Abbreviations: tBHP, *tert*-butylhydroperoxide; MDA, malondialdehyde; GSH, reduced glutathione; GPT, glutamic-pyruvate transaminase; AP, alkaline phosphatase; I, andrographolide; II, andrographiside; III, neoandrographolide.

Table 1. Effect of the diterpenes (I, II and III) and silymarin on hepatic lipid peroxidation in mice after CCl_4 and tBHP intoxication

Group	MDA formation (nmol/g liver/10 min)			
	CCl_4	Inhibition (%)	tBHP	Inhibition (%)
Control	131 ± 4.5		150 ± 6.0	
Toxin-treated	159 ± 13.8*†	—	216 ± 19*†	—
I-toxin-treated	108 ± 11.6*‡§	32	170 ± 29*‡§	21
II-toxin-treated	103 ± 10*‡	35	141 ± 10*‡	35
III-toxin-treated	96 ± 11.7*‡	40	132 ± 17*‡	39
Silymarin-toxin-treated	94.5 ± 6.1*‡	41	131 ± 5.0*‡	40

Data represent mean ± SD of five animals in each group.
* $P < 0.05$.
† Comparison with control group.
‡ Comparison with respective toxin-treated group.
§ Comparison with respective silymarin-toxin-treated group.

concentration was chromatographed over a column of silica gel (two and a half times its weight) and eluted with chloroform containing 2% methanol to afford a further crop of III (0.9 g). Subsequent elution with a chloroform methanol (19:1) mixture yielded II as needles (2.2 g), m.p. 192–193°. The diterpenes thus obtained were of high purity as checked by their melting points, optical rotations, TLC (single spot), IR and ^1H NMR data which corresponded to those described in literature.

Silymarin was provided by Gruppo Inverni Della Beffa (Milan, Italy). tBHP was procured from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals used were of LR grade and obtained from reputed Indian firms.

The present study was conducted on male Swiss mice (20–22 g) which were obtained from the Animal House of the laboratory and kept in plastic cages bedded with rice-husk. They were fed a stock pellet diet *ad lib.* and allowed free access to drinking water. They were kept on a 12/12 hr light/dark cycle.

The crystals of the diterpenes were ground in a mortar to a fine powder. Suspensions of I, II, III and silymarin were then prepared in olive oil (2 mg/0.2 mL) and injected i.p. to mice at a dose of 100 mg/kg for 3 consecutive days. Eight hours after the last administration animals were given a single i.p. dose of CCl_4 (10 μL) or tBHP (15 μL) in 0.2 mL olive oil. Control mice only received the vehicle. Animals were then fasted and 17 hr after intoxication, they were anaesthetized with ether to collect their blood from retro-orbital sinus. Serum was separated by centrifugation after keeping the samples at 10° for 30 min. After blood collection, the animals were killed by cervical dislocation, livers were excised, perfused with chilled normal saline and weighed. Liver homogenates (25%, w/v) were prepared in ice-cold 50 mM potassium phosphate buffer, pH 7.4. These homogenates were used for the estimation of GSH by the method of Moron *et al.* [14] and assayed for the MDA formation as described in the method of Wills [15]. The activity of alkaline phosphatase (AP) was measured by the method of Bessey *et al.* [16] and GPT was estimated in accordance with the procedure given by Reitman and Frankel [17]. Proteins were determined according to the method of Lowry *et al.* [18] using bovine serum albumin as a standard.

Statistical significance of the difference among different groups was analysed by Student's unpaired *t*-test.

Results and Discussion

Table 1 demonstrates the effect of the diterpenoid constituents I, II and III of *A. paniculata* on hepatic lipid

Table 2. Effect of the diterpenes (I, II and III) and silymarin on hepatic GSH status in mice after CCl_4 and tBHP intoxication

Group	GSH (μmol/g liver)			
	CCl_4	Depletion (%)	tBHP	Depletion (%)
Control	7.1 ± 0.43		7.7 ± 0.67	
Toxin-treated	4.5 ± 0.48*†	37	5.4 ± 0.42*†	29
I-toxin-treated	5.6 ± 0.62*†‡§	21	6.3 ± 0.77*†‡§	18
II-toxin-treated	6.5 ± 0.49*†‡§	8	6.6 ± 0.45*†‡§	14
III-toxin-treated	7.4 ± 0.32*‡	—	6.9 ± 0.41*†‡	10
Silymarin-toxin-treated	7.5 ± 0.41*‡	—	7.4 ± 0.48*‡	4

Legends showing different comparisons and P value are the same as for Table 1.

peroxidation in mice after CCl_4 and tBHP challenge and their comparison with silymarin. The thiobarbituric acid reaction with MDA is generally considered to be an indicator of the secondary breakdown products of oxidized polyunsaturated fatty acids. As can be observed, hepatic MDA formation was significantly increased in both CCl_4 and tBHP treated mice. However, intraperitoneal administration of I, II and III inhibited hepatic MDA formation, respectively, by 32%, 35% and 40% in CCl_4 treated mice and by 21%, 35% and 39% in tBHP treated animals. On comparing the protective effect of the diterpenes on hepatic lipid peroxidation with that of silymarin, it was noticed that I was less effective whereas II and III offered protection which was comparable to that of silymarin in both of the toxic models.

Intoxication with CCl_4 and tBHP caused significant depletion in GSH contents in the liver of mice (Table 2). However, pretreatment with I and II prevented the GSH depletion by 16% and 29% in CCl_4 treated group and by 11% and 15% in tBHP treated group, respectively. GSH was restored to normal tissue levels only by III and the effect produced was comparable to that by silymarin in both of the treated groups.

The data in Table 3 show that the administration of these plant products to mice led to a significant inhibition of the release of GPT and AP in the intoxicated animals. Only II and III reversed the elevated levels to the same extent as silymarin in the CCl_4 or tBHP administered groups.

Our observation of increased cytotoxicity in CCl_4 and tBHP treated animals with respect to lipid peroxidation, GSH depletion and release of serum GPT and AP confirms that the mechanism by which these toxins produce hepatic necrosis involves metabolic conversion of the drug into reactive electrophilic species which may either: (i) abstract hydrogen atoms from lipids forming carbon-centred lipid radicals to propagate the peroxidation process [19, 20] or (ii) conjugate with hepatic GSH to produce GSH depletion followed by the appearance of cellular damage [21] which is indicated by the elevated levels of enzymes in the serum [22, 23].

The investigation reported in the paper employed administration of the plant products for 3 days prior to the hepatotoxin challenge. The selection of the schedule was based on data from studies on serum markers of hepatotoxicity. The administration of the three diterpenes (I, II and III) and silymarin according to this regime afforded protection against lipid peroxidation, GSH depletion and leakage of GPT and AP in both CCl_4 and tBHP induced toxic models thus showing a broad range of their inhibitory capacities. Our finding of inhibition in GPT and AP levels in the serum following administration of I is in agreement with earlier studies reporting that treatment with I normalized serum indices of toxicity induced by CCl_4, galactosamine or paracetamol [7, 24]. The inhibitory effect on MDA formation which has been related to the membrane-stabilizing or free radical-quenching activities of the reference hepatoprotective principle silymarin [25] revealed the antioxidant potential of these diterpenes (I, II and III). Earlier investigations attributed this effect to inhibition by the diterpenes of components of the cytochrome P450 system [8]. However, the results of the present work demonstrate a relationship of the structure of the diterpenoid constituents with their inhibitory activity. Although earlier reports have attributed the protective action of I to stimulation of hepatic regeneration [24], the present study suggests that it is due to antioxidant action like that of other plant products [26, 27]. The fact that II and III showed better protection than I against CCl_4 and tBHP induced toxicity indicated that the glucosidic moiety in these molecules might act as powerful antioxidant thereby enhancing the rapid inactivation of the liver damaging species.

The present biochemical data reveal the hepatoprotective

Table 3. Effect of the diterpenes (I, II and III) and silymarin on serum GPT and AP levels in mice after CCl_4 and tBHP intoxication

Group	GPT (U/L)				AP (U/L)			
	CCl_4	Inhibition (%)	tBHP	Inhibition (%)	CCl_4	Inhibition (%)	tBHP	Inhibition (%)
Control	34 ± 1.2		36 ± 3.7		331 ± 25		266 ± 22	
Toxin-treated	134 ± 10*†	—	76 ± 0.4*†	—	737 ± 35*†	—	387 ± 15*†	—
I-toxin-treated	119 ± 8*‡§	11	63 ± 8*‡	17	596 ± 37*‡§	19	323 ± 30*‡§	17
II-toxin-treated	110 ± 10*‡	18	59 ± 4.8*‡	22	569 ± 54*‡	23	290 ± 38*‡	25
III-toxin-treated	111 ± 10*‡	17	54 ± 4.8*‡	29	536 ± 37*‡	27	280 ± 35*‡	28
Silymarin-toxin-treated	104 ± 12*‡	22	55 ± 3.6*‡	28	511 ± 51*‡	31	286 ± 22*‡	26

Legends showing different comparisons and P value are the same as for Table 1.

activity of I, II and III against two well-known hepatotoxicants i.e. CCl_4 and tBHP, which is consistent with the previously reported histopathological results [7] showing that diterpene pretreatment ameliorates toxin-induced histopathological alterations in the livers of experimental animals.

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