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Picroliv, picroside-I and kutkoside from *Picrorhiza kurrooa* are scavengers of superoxide anions

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Abstract—Picroliv, the active principle of *Picrorhiza kurrooa*, and its main components which are a mixture of the iridoid glycosides, picroside-I and kutkoside, were studied *in vitro* as potential scavengers of oxygen free radicals. The superoxide (O_2^-) anions generated in a xanthine–xanthine oxidase system, as measured in terms of uric acid formed and the reduction of nitroblue tetrazolium were shown to be suppressed by picroliv, picroside-I and kutkoside. Picroliv as well as both glycosides inhibited the non-enzymic generation of O_2^- anions in a phenazine methosulphate NADH system. Malonaldehyde (MDA) generation in rat liver microsomes as stimulated by both the ascorbate–Fe^{2+} and NADPH–ADP–Fe^{2+} systems was shown to be inhibited by the Picroliv glycosides. Known antioxidants tocopherol (vitamin E) and butylated hydroxyanisole (BHA) were also compared with regard to their antioxidant actions in the above system. It was found that BHA afforded protection against ascorbate–Fe^{2+}-induced MDA formation in microsomes but did not interfere with enzymic or non-enzymic O_2^- anion generation; and tocopherol inhibited lipid peroxidation in microsomes by both prooxidant systems and the generation of O_2^- anions in the non-enzymic system but did not interfere with xanthine oxidase activity. The present study shows that picroliv, picroside-I and kutkoside possess the properties of antioxidants which appear to be mediated through activity like that of superoxide dismutase, metal ion chelators and xanthine oxidase inhibitors.

Picrorhiza kurrooa (Scrophulariaceae, regional name Kutki) forms an ingredient of many Indian herbal preparations used for the treatment of liver ailments. These plants are prolific in north India [1]. In the present study *P. kurrooa* was collected from Garhwal district of U.P. and identified pharmacognistically. Picroliv is the standard preparation containing mainly a mixture of two iridoid glycosides, picroside-I and kutkoside (1:1.5 w/w) obtained from the ethanolic extract of the roots and rhizomes of *P. kurrooa* (Fig. 1). The picroside-I and kutkoside mixture accounts for approximately 60% of the constituents of picroliv. The remainder is a mixture of unidentified compounds. Picroliv has been reported to be a potent hepatoprotective agent against various hepatotoxins including hepatitis B virus [2–7]. Picroliv restores choleretic activity during thioacetamide-induced hepatic damage in experimental animals by increasing the biosynthesis and secretion of bile acids [8].

The protective effects have been ascribed to a membrane-stabilizing action which prevents hepatocellular necrosis [9]. Recent observations showed that administration of picroliv in *Plasmodium berghei*-infected *Mastomys natalensis* (African desert rat) significantly reduced the elevated levels of hepato-specific enzymes, lipid peroxides and lipid hydroperoxides (including several biochemical markers of tissue injury), and facilitated the recovery of hepatic superoxide dismutase, reduced glutathione levels and the activities of glutathione-related enzymes [10, 11]. The hepatotoxic action of *P. berghei* infection is thought to be mediated through the generation of free radicals responsible for non-specific peroxidation of lipids and membrane damage [12]. Recently, we have reported high density lipoprotein to be a scavenger of superoxide (O_2^-) anions [13]. The purpose of the present study was to demonstrate the free radical-scavenging property of picroliv and its components against the generation of O_2^- anions both in enzymic and non-enzymic systems *in vitro*. The effect of picroliv as a protector of O_2^- anions and lipid peroxidation in microsomes has been compared with that of tocopherol (vitamin E) and butylated hydroxyanisole (BHA*).

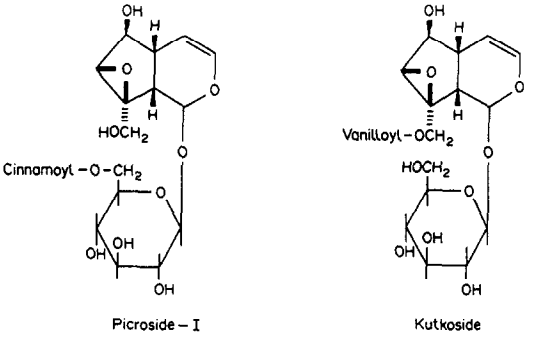

Fig. 1.

Materials and Methods

Xanthine oxidase (EC 1.1.3.22), NADH and nitroblue tetrazolium (NBT) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.) and other chemicals used were of analytical grade. Picroliv, picroside-I and kutkoside were procured from the Medicinal Chemistry Division of our Institute.

The effect of test substances on the generation of O_2^- anions in the enzymic system of xanthine–xanthine oxidase was investigated [14]. Xanthine oxidase activity in a solution containing 80 μM xanthine in 0.1 M, phosphate buffer pH 7.4, 0.03 U/mL of xanthine oxidase added with known concentration of test substances (picroliv, picroside-I kutkoside, tocopherol and BHA) to a final volume of 2.5 mL, was assayed spectrophotometrically at 295 nm. The change in extinction/min was compared with the reaction mixture which did not include the test substances. The influence of test samples on NBT reduction by O_2^-

* Abbreviations: NBT, nitroblue tetrazolium; MDA, malonaldehyde; BHA, butylated hydroxyanisole.

anions was measured in the reaction mixture containing 0.03 U/mL of xanthine oxidase, 80 μM of xanthine and 160 μM NBT in 0.1 M phosphate buffer pH 7.4. The reaction was stopped by adding glacial CH_3COOH and extinction was read at 560 nm against respective blanks.

Another system employed for the non-enzymic generation of O_2^- anions was comprised of 10 μM phenazine methosulphate, 80 μM NADH and 40 μM NBT in 0.1 M pyrophosphate buffer pH 9.2 to a final volume of 2.5 mL [15]. After 90 sec incubation in the presence of picroliv or other test samples, the reaction was terminated by adding 1.0 mL CH_3COOH and colour was read at 560 nm against respective reference tubes. Boiled rat liver microsomes (2 mg protein), 20 mM sodium ascorbate and 2 mM $FeSO_4.7H_2O$ were added with known concentrations of test samples in 0.1 M phosphate buffer pH 7.4 to a final volume of 2.5 mL. The reaction mixture was incubated at 37° for 90 min. Similarly, separate tubes containing microsomes, sodium ascorbate or Fe^{2+} and various concentrations of test samples were incubated and mixed together by test sample to serve as corresponding references. Malonaldehyde (MDA) content in both sets was measured by the thiobarbituric acid method [16]. The influence of tested substances on the formation of lipid peroxides was calculated after subtracting the values of reference tubes from those of their respective experimental tubes. The effect of picroliv or other test samples on MDA formation by freshly prepared rat liver microsomes in which lipid peroxidation had been stimulated by 100 μM NADPH, 500 μM ADP and 2 mM $FeSO_4$ was measured as detailed above. IC_{50}, slope and correlation coefficient were calculated by regression analysis on *X*-axis, log concentration vs *Y*-axis, % inhibition [17].

Results and Discussion

Enzymic generation of superoxide anions. The enzymic oxidation of xanthine to uric acid was inhibited to varying extents by picroliv, picroside-I and kutkoside in a concentration-dependent manner. The activity of picroside-I was greater (IC_{50} 69.9 μM) than that of picroliv and kutkoside (Table 1). However, BHA and tocopherol failed to interfere with xanthine oxidase activity within the tested ranges. It can be seen that picroliv and the tested iridoid glycosides suppressed the generation of O_2^- anions in the xanthine–xanthine oxidase system as measured by the reduction of NBT; however, these effects were less than those of tocopherol. BHA did not show any activity in the above system (Table 2). The data showed that picroliv and its glycosides are inhibitors of xanthine oxidase as well as scavenger of O_2^- anions. Recently, it has been reported that the antioxidant activity of propranolol may be due to its inhibitory action on xanthine oxidase [18].

Non-enzymic generation of superoxide anions. Picroside-I trapped the O_2^- anions, generated by NADH and phenozine methosulphate and responsible for the reduction of NBT in the reaction mixture, more effectively than picroliv or kutkoside (Table 3). However, these glycosides were less active inhibitors than tocopherol. BHA did not interfere with the generation of O_2^- anions in the above system. The free radical-scavenging property of a compound may be correlated with its hepatoprotective activity. The flavonoids which are scavengers of O_2^- anions showed a hepatoprotective action by their selective binding to hepatocytes [19].

Enzymic and non-enzymic lipid peroxidation in microsomes. Both iridoid glycosides and their mixture, when added to rat liver microsomes in which lipid peroxidation was induced enzymatically by NADPH–ADP–Fe^{2+}, suppressed the formation of MDA (Table 4). The antioxidant action of picroside-I (IC_{50} 232.5 μM) was greater than that of picroliv or kutkoside. However, tocopherol was more active than the glycosides, but BHA did not inhibit MDA formation within the tested concentrations.

Table 1. Inhibition of xanthine oxidase

Substance tested	IC_{50} (μM)	*b*	*r*	N
Picroliv	81.5 ± 1.0*	26.1	0.99	7
Picroside-I	69.9 ± 4.0	33.7	0.84	7
Kutkoside	354.6 ± 28.8	22.1	0.96	11
Tocopherol	Inactive at 10–500 μM	—	—	12
BHA	Inactive at 20–400 μM	—	—	10

* mg/L.

Assays were performed as described in Materials and Methods.

IC_{50} values were calculated from regression lines where *x* was log of tested substance concentration and *y* was per cent inhibition of enzyme activity.

b, stops of regression line.

N, number of experiments.

Table 2. Reduction of NBT by superoxide anions in the xanthine–xanthine oxidase system

Substance tested	IC_{50} (μM)	*b*	*r*	N
Picroliv	158.2 ± 5.6*	25.0	0.98	7
Picroside-I	67.9 ± 4.4	32.8	0.81	7
Kutkoside	291.5 ± 18.5	23.0	0.98	12
Tocopherol	53.2 ± 4.3	28.9	0.94	10
BHA	Inactive at 20–400 μM	—	—	10

* mg/L.

Assays were performed as described in Materials and Methods.

IC_{50} values were calculated as in Table 1.

Table 3. Inhibition of non-enzymic generation of O_2^- anions by phenozine methosulphate and NADH

Substance tested	IC_{50} (μM)	*b*	*r*	N
Picroliv	85.3 ± 8.3*	25.9	0.99	7
Picroside-I	85.5 ± 5.5	30.3	0.93	6
Kutkoside	372.3 ± 8.8	22.0	0.96	11
Tocopherol	116.2 ± 5.6	24.2	0.96	8
BHA	Inactive at 20–400 μM	—	—	10

* mg/L.

Assays were performed as described in Materials and Methods.

IC_{50} values were calculated as in Table 1.

Table 5 shows that picroside-I was again a more potent protector than picroliv or kutkoside against the formation of MDA in microsomes stimulated non-enzymatically by ascorbate and Fe^{2+} (IC_{50} 26.6 μM). BHA possessed a greater potential for neutralizing the reaction of non-enzymic lipid peroxidation than α-tocopherol or the glycosides. Free radicals are believed to be involved in various human disease processes. One direct consequence

Table 4. Inhibition of MDA generation by enzymic lipid peroxidation in rat liver microsomes stimulated by NADPH, ADP and iron

Substance tested	IC_{50} (μM)	*b*	*r*	N
Picroliv	120.3 ± 7.8*	24.0	0.99	7
Picroside-I	126.6 ± 12.3	27.9	0.92	6
Kutkoside	366.9 ± 13.9	22.0	0.96	13
Tocopherol	263.3 ± 17.1	20.6	0.97	10
BHA	Inactive at 20–400 μM	—	—	10

* mg/L.

Assays were performed as described in Materials and Methods.

IC_{50} values were calculated as in Table 1.

Table 5. Inhibition of formation of MDA by non-enzymic lipid peroxidation in boiled rat liver microsomes stimulated by ascorbate and iron

Substance tested	IC_{50} (μM)	*b*	*r*	N
Picroliv	173.8 ± 5.6*	22.3	0.98	10
Picroside-I	232.5 ± 11.5	24.3	0.99	10
Kutkoside	561.5 ± 27.4	20.3	0.99	15
Tocopherol	141.9 ± 8.4	23.2	0.97	9
BHA	17.7 ± 0.5	40.0	0.98	9

* mg/L.

Assays were performed as described in Materials and Methods.

IC_{50} values were calculated as in Table 1.

of the attack of O_2^- anions or $OH^\cdot$ free radicals is the peroxidative breakdown of membrane lipids which has been shown to play an important role in liver damage [20]. In the *in vitro* systems selected by us, lipid peroxidation is thought to be mediated by the reduction of iron to ferrous state which catalysed the decomposition of fatty acid free radicals into MDA [21]. Picroliv and iridoid glycosides may play a dual role by chelating Fe^{2+} ions and scavenging the free radicals from fatty hydroperoxides so as to inhibit the reaction of lipid peroxidation. The only system in which BHA, a synthetic antioxidant, showed similar properties to that of the investigated substances, is the non-enzymatic oxidation of lipids as shown by the suppression of MDA formation in microsomes. We believe that the antioxidant property of picroliv and its components is due to their aglycon (iridoid) as well as glycoside moieties and the potential of this activity may be affected by esterification of the labile hydroxyl group. Our observations suggest that picroside-I containing a cinnamoyl group is a more potent O_2^- anion scavenger than kutkoside which has a vanilloyl group (Fig. 1). Furthermore, the chloroform-extractable fraction of *P. kurrooa* containing picroside-II, which has structural similarity to picroside-I after replacement of the cinnamoyl group with vanilloyl, possesses a lower liver protective action than that of the ethanol-extractable fraction enriched with picroside-I [22]. A higher slope value (*b*) and correlation coefficient (*r*) of picroside-I than other tested glycosides confirm its greater potential for free radical scavenging. Silymarin, a flavolignan and hepatoprotective agent [23], also possesses an antioxidant action to prevent pathological lesions due to lipid peroxidation during CCl_4 administration [24].

The antioxidant property of picroliv, picroside-I and kutkoside depends on preventing the formation of free radicals at the level of O_2^- anions, possibly acting like superoxide dismutase, xanthine oxidase inhibitors and metal ion chelators. The mechanism of antioxidant action of these iridoid glycosides has some similarities to that of tocopherol, but is entirely different from that of synthetic antioxidants like BHA which seem to be "low level" scavengers of hydroxyl free radicals. The hepatoprotective action of picroliv glycosides may be due to the prevention of lipid peroxidation and free radical generation during liver damage. Picroside-I was a more potent O_2^- anions scavenger than picroliv or kutkoside.

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Detection of human lung cytochromes P450 that are immunochemically related to cytochrome P450IIE1 and cytochrome P450IIIA

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Abstract—We have used monoclonal antibodies that were prepared against and specifically recognize human hepatic cytochromes P450 as probes for solid phase radioimmunoassay and Western immunoblotting to directly demonstrate the presence in human lung microsomes of cytochromes P450 immunochemically related to human liver cytochromes P450IIE1 (CYP2E1) and P450IIIA (CYP3A). The detected levels of these cytochromes are much lower than levels in human liver microsomes, but similar to the levels seen in microsomes from untreated baboon lung. Proteins immunochemically related to two other constitutive hepatic cytochromes P450, cytochrome P450IIC8 (CYP2C8) and cytochrome P450IIC9 (CYP2C9), were not detectable in lung microsomes.

The characterization of cytochrome P450 in the human lung, and particularly of individual cytochromes P450, is of interest to many pharmacologists and toxicologists because of the role these enzymes play in the bioactivation of numerous xenobiotics to cytotoxic or mutagenic electrophiles [1–5]. However, in contrast to the relatively detailed knowledge concerning individual cytochromes P450 in animal lung, individual forms in the human lung have not been extensively characterized until recently. Immunochemical and metabolic studies have identified a CYP1A1* species in human lung microsomes [7]. Antibodies that recognize rat liver CYP2B1, CYP2B2 and CYP1A2, and human liver CYP3A3 were used to seek immunochemically related forms in human lung microsomes; in all cases, no related cytochrome was

* Individual cytochromes P450 are abbreviated using the "CYP" nomenclature suggested by Nebert *et al.* [6]. In all cases, these abbreviations refer to enzyme protein, and not to genetic loci.