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Efficient Utilization of Licorice Root by Alkaline Extraction

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Abstract. *Compared to studies of water extracts of plants, those utilising alkaline extracts are limited. Both water and alkaline extracts from licorice root were compared regarding their biological activities. Licorice root was successively extracted first with water or alkaline solution (pH 9 or 12), and the alkaline (pH 12.0) extract was further separated into 50% ethanol-soluble and -insoluble fractions. Viable cell number was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Antibacterial activity against Porphyromonas gingivalis 381 was determined by turbidity assay. Cytochrome P-450 (CYP)3A4 activity was measured by β-hydroxylation of testosterone using human recombinant CYP3A4. Radical intensity of superoxide and hydroxyl radicals was determined by electron spin resonance spectroscopy. Alkaline extraction yielded slightly higher amounts of dried materials compared to water extraction. Alkaline extract showed higher anti-HIV and antibacterial activities, and similar magnitudes of CYP3A4 inhibitory and superoxide and hydroxyl radical-scavenging activities, compared to water extract. When alkaline extract was fractionated by 50% ethanol, anti-HIV activity was recovered from the insoluble fraction representing approximately 3% of the alkaline extract, whereas antibacterial activity was concentrated in the soluble fraction rich in glycyrrhizid acid, flavanones and chalcones. All extracts and sub-fractions led to bimodal hormetic dose-response (maximum hormetic response=238%) on the bacterial growth. The present study demonstrated the superiority of alkaline extraction over water extraction for preparing anti-HIV and antibacterial agents at higher yield from licorice root.*

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We have reported that lignin–carbohydrate complex fractions prepared by acid precipitation of the alkaline extracts of pine cone, pine seed shell, catuaba bark, cacao husk, cacao mass, *Lentinus* edodes mycelia potently protected cells from HIV-infection [selectivity index (SI)=7-311] (1), and from UV irradiation (SI=7.6-38.1) (2). Similarly, crude alkaline extract of the leaves of *Sasa senanensis* Rehder showed comparable anti-HIV (SI=36-45) and anti-UV activity (SI=20-39) with lignin-carbohydrate complex fractions (3). On the other hand, hot-water extracts of a total of ten Kampo medicines and their twenty-five constituent plants had much lower anti-HIV (SI=1-8) and anti-UV activity (SI=1-4.4) (4). This raises the possibility that the use of alkaline extraction is more advantageous than hot-water extraction at obtaining higher amounts of anti-HIV and anti-UV substances. However, this possibility has not yet been tested with water and alkaline extracts prepared from the same plant species. To clarify this point, we recently performed a comparative study with both water and alkaline extracts of green tea leaf, oolong tea leaf and orange flower, and found that water extracts had higher antibacterial, CYP3A4-inhibitory and superoxide-scavenging activities, whereas alkaline extract had higher anti-HIV and hydroxyl radical-scavenging activity (5).

In order to confirm the usefulness of alkaline extraction, herein we performed a similar comparative study with water and alkaline (pH 9.0 and pH 12.0) extracts of licorice root (*Glycyrrhiza glabra*) (Figure 1). In the present study, we also compared the biological activities of two subfractions of alkaline (pH 12.0) extracts: the 50% ethanol-soluble fraction (that contained higher amounts of flavones, chalcones and triterpenoid saponin) and the 50% ethanol-insoluble fraction (that is expected to contain higher amounts of lignin-carbohydrate complex) (Figures 2-4).

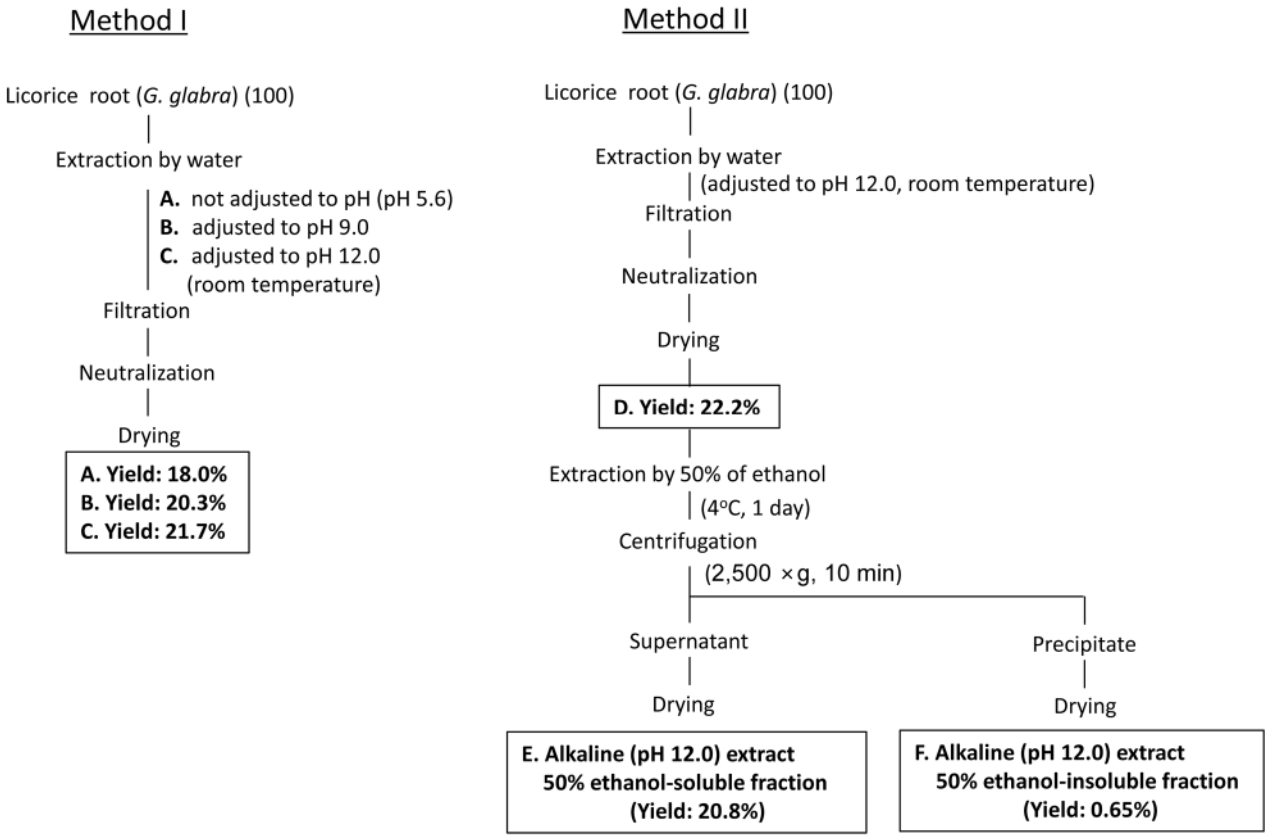

Figure 1. *Fractional preparation of water and alkaline extracts from licorice root (Glycyrrhiza glabra).*

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle's medium (DMEM): Gibco BRL, Grand Island, NY, USA; fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), hypoxanthine (HX), xanthine oxidase (XOD), diethylene-triaminepenta-acetic acid (DETAPAC), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO): Dojin, Kumamoto, Japan; RPMI–1640 medium, azidothymidine (AZT), 2',3'-dideoxycytidine (ddC), gallic acid: Sigma-Aldrich Co. St. Louis, MO, USA; dimethyl sulfoxide (DMSO), dextran sulfate (5 kDa): Wako Pure Chemical Ind., Ltd., Osaka, Japan; sodium ascorbate: Tokyo Chemical Industry Co., Ltd., Tokyo, Japan; curdlan sulphate, 79 kDa: Ajinomoto Co. Inc., Tokyo, Japan.

Preparation of water and alkaline extracts. Licorice roots (*G. glabra* harvested in Afghanistan) were extracted for 20 h with water (not adjusted or adjusted to pH 9 or pH 12) at room temperature, filtered through a membrane filter (pore size: 5 μm), neutralized with NaOH or H_2SO_4 and then dried to obtain the water extract (A), alkaline (pH 9.0) extract (B) and alkaline (pH 12.0) extract (C) at yields of 18.0%, 20.3% and 21.7%, respectively (Method I in Figure 1).

Licorice root was also extracted for 23 h with water (adjusted to pH 12) at room temperature, filtered, neutralized and then dried to obtain alkaline (pH 12.0) extract (D) (yield: 22.2%). This alkaline extract was further extracted with 50% ethanol (4˚C, 24 h), centrifuged to separate into supernatant and precipitate, and then dried to obtain the 50% ethanol-soluble (E) and 50% ethanol-insoluble (F) fractions at yields of 20.8% and 0.65%, respectively (Method II in Figure 1).

Ultra performance liquid chromatography (UPLC) separation of water and alkaline extracts. Each extract (25 mg) was prepared to 25 ml with 50% ethanol, filtered through a membrane filter (0.2 μm), and then 2 μl was applied to ACQUITY UPLC, using the following analytical conditions: (i) column, ACQUITY UPLC BEH C18 (1.7 μm, 150 mm × 2.0 mm i.d.)(Waters Corporation, Milford, MA, USA); (ii) mobile phase was 0.1% aq. formic acid (A) and MeCN (B), 0-0.5 min A:B=80:20, 0.5-17.5 min A:B=80:20→30:70, 17.5-18.5 min A:B=0:100, 18.5-21.0 min A:B=80:20; (iii) flow rate, 0.3 ml/min; (iv) detection, Photodiode Array Detector (Waters Corporation) eλ (254 nm 316 nm); (v) column temperature, 40˚C; (vi) sample maintenance temperature, 25˚C. Standard compounds used were liquiritin apioside, liquiritigenin 7-apiosylglucoside, liquiritin, neoliquiritin, liquiritigenin, isoliquiritin apioside, licurazid, isoliquiritin, neoisoliquiritin, isoliquiritigenin and glycyrrhizic acid (provided by Maruzen Pharmaceuticals Co. Ltd., Hiroshima, Japan) (Figure 2).

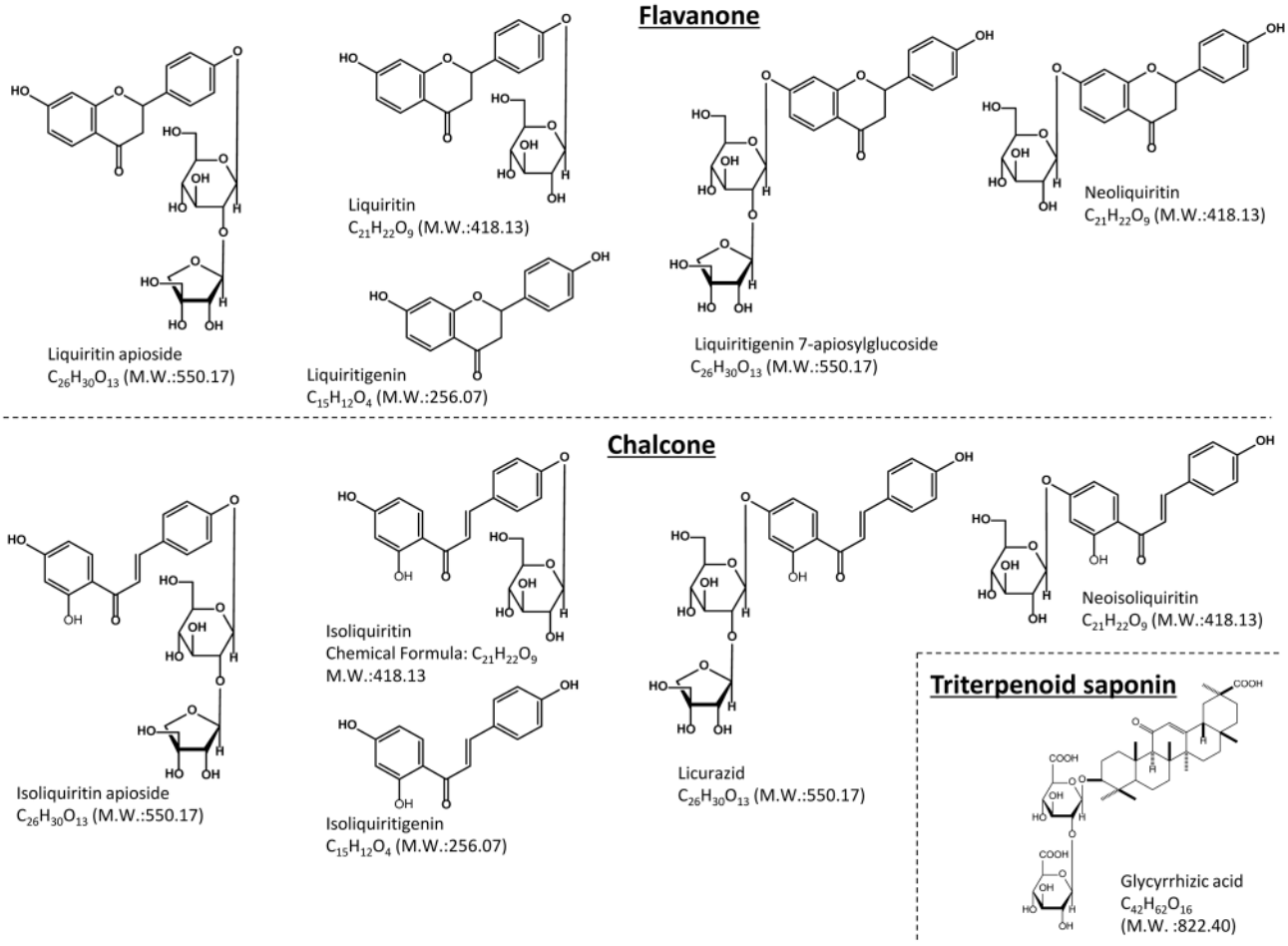

Figure 2. *Structure of five flavanones, five chalcones and one triterpenoid saponin, used as standards for separation by ultra performance liquid chromatography (see Figure 3) and high performance liquid chromatography (see Figure 4).*

Assay for anti-HIV activity. Human T-cell leukemia virus I (HTLV-I)-bearing CD4- positive human T-cell line MT-4 (supplied by Dr. Naoki Yamamoto) was cultured in RPMI-1640 medium supplemented with 10% FBS and infected with HIV-1_{IIIB} at a multiplicity of infection of 0.01. HIV- and mock-infected MT-4 cells (3×10^4 cells/96-microwell) were incubated for five days with different concentrations of extracts and the relative viable cell number was determined by MTT assay. The concentration that reduced the viable cell number of the uninfected cells by 50% (CC_{50}) and the concentration that increased the viable cell number of the HIV-infected cells to the 50% that of control (mock-infected, untreated) cells (EC_{50}) were determined from the dose–response curve with mock-infected and HIV-infected cells, respectively. The anti-HIV activity was evaluated by the selectivity index (SI), which was calculated using the following equation: $SI=CC_{50}/EC_{50}$ (6).

Assay for antibacterial activity. Porphyromonas gingivalis 381 (1×10^6 cfu/ml) was incubated for 24, 27, 30 or 42 h at 37˚C in Gifu Anaerobic Medium (GAM) containing containing serially diluted samples, 5 μg/ml hemin and 1 μg/ml menadione under anaerobic conditions with mixed gas of nitrogen (83%), hydrogen (7%) and CO_2 (10%), and then the absorbance at 595 nm of the bacterial suspension was measured (7). From the dose–response curve, the concentration that reduced the bacterial growth by 50% (IC_{50}) was determined.

Measurement of CYP3A4 activity. CYP3A4 activity was measured by β-hydroxylation of testosterone using human recombinant CYP3A4 (8, 9). The reaction mixture, containing 200 mM potassium phosphate buffer (pH 7.4), NADPH regenerating system (1.3 mM NADPH, 1.3 mM glucose-6-phosphate, 0.2 U/ml glucose-6-phosphate dehydrogenase, and 3.3 mM $MgCl_2$) along with 0, 10, 30, 100, 300, 600 and 1000 μg/ml of the test extract or vehicle in triplicate and the human recombinant CYP3A4 (16.5 pmol/ml), was preincubated at 37˚C for 5 min. The reaction was started by the addition of 300 μM testosterone substrates. The final volume of the reaction mixture was 250 μl with a final DMSO concentration of 0.5%. The reaction was stopped by the addition of 500 μl ethyl acetate after 15 min. After centrifugation (15,000 × *g*, 5 min), 400 μl of supernatant was collected, dried, and resuspended in 100 μl of

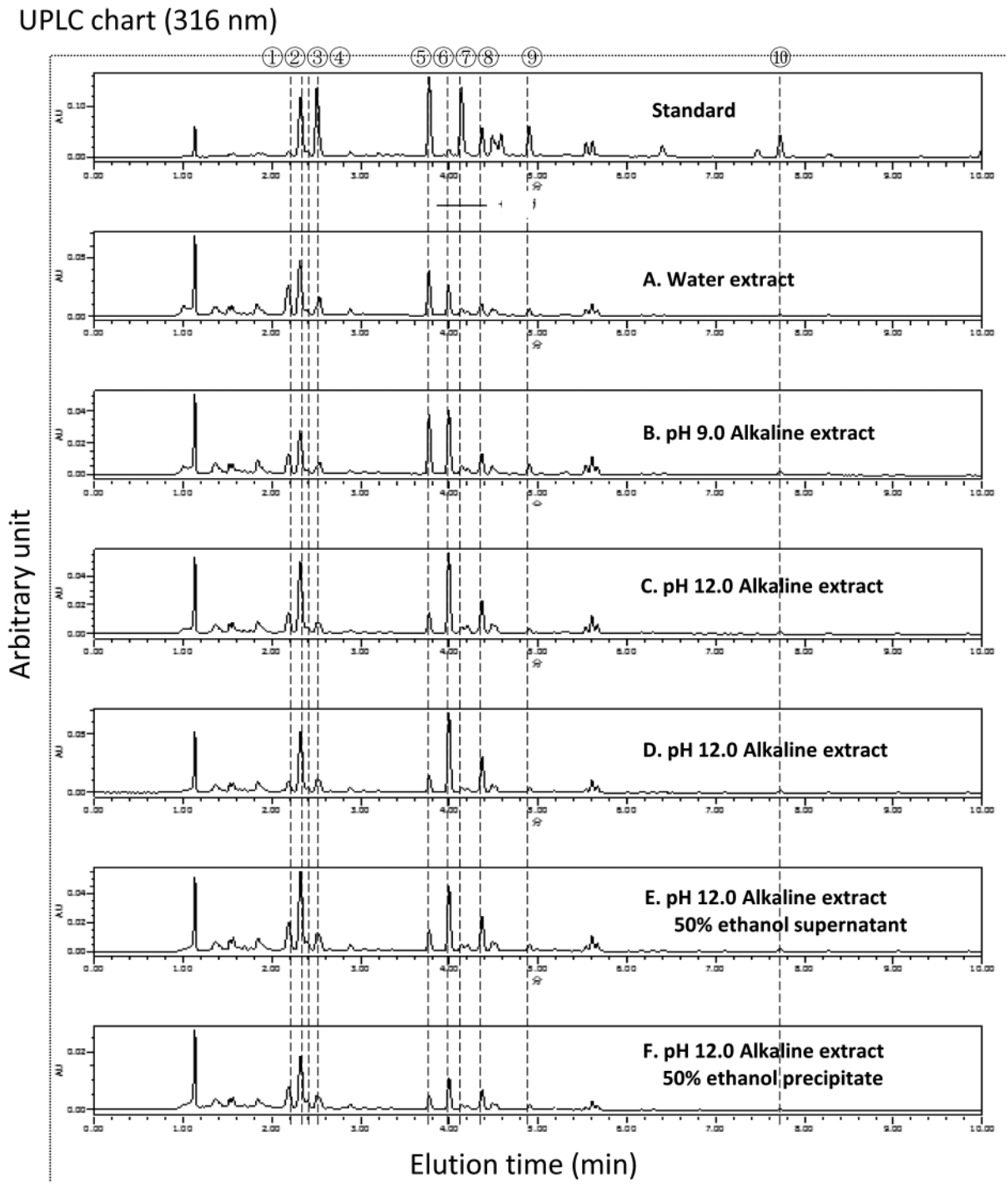

Figure 3. *Ultra performance liquid chromatography separation of 10 standards: 1: liquiritin apioside, 2: liquiritigenin 7-apiosylglucoside, 3: liquiritin, 4: neoliquiritin, 5: liquiritigenin, 6: isoliquiritin apioside, 7: licurazid, 8: isoliquiritin, 9: neoisoliquiritin, 10: isoliquiritigenin; water extract (A), pH 9.0 alkaline extract (B), pH 12.0 alkaline extract (C), pH 12.0 alkaline extract (D) and 50% ethanol-soluble (E) and -insoluble (F) fractions of D.*

methanol. Analyses of the metabolites were performed by HPLC (JASCO PU2089, AS2057, UV2075 ChromNAV) equipped with TSKgel ODS-120A, 4.6 mmID×25 cm, 5 μm column (TOSOH, Tokyo, Japan). The mobile phase consisted of 70% methanol and 30% water. The metabolites were separated using an isocratic method at a flow rate of 1.0 ml/min. Quantification of the metabolites was performed by comparing the HPLC peak area at 254 nm to that of 11α-progesterone, the internal standard. The

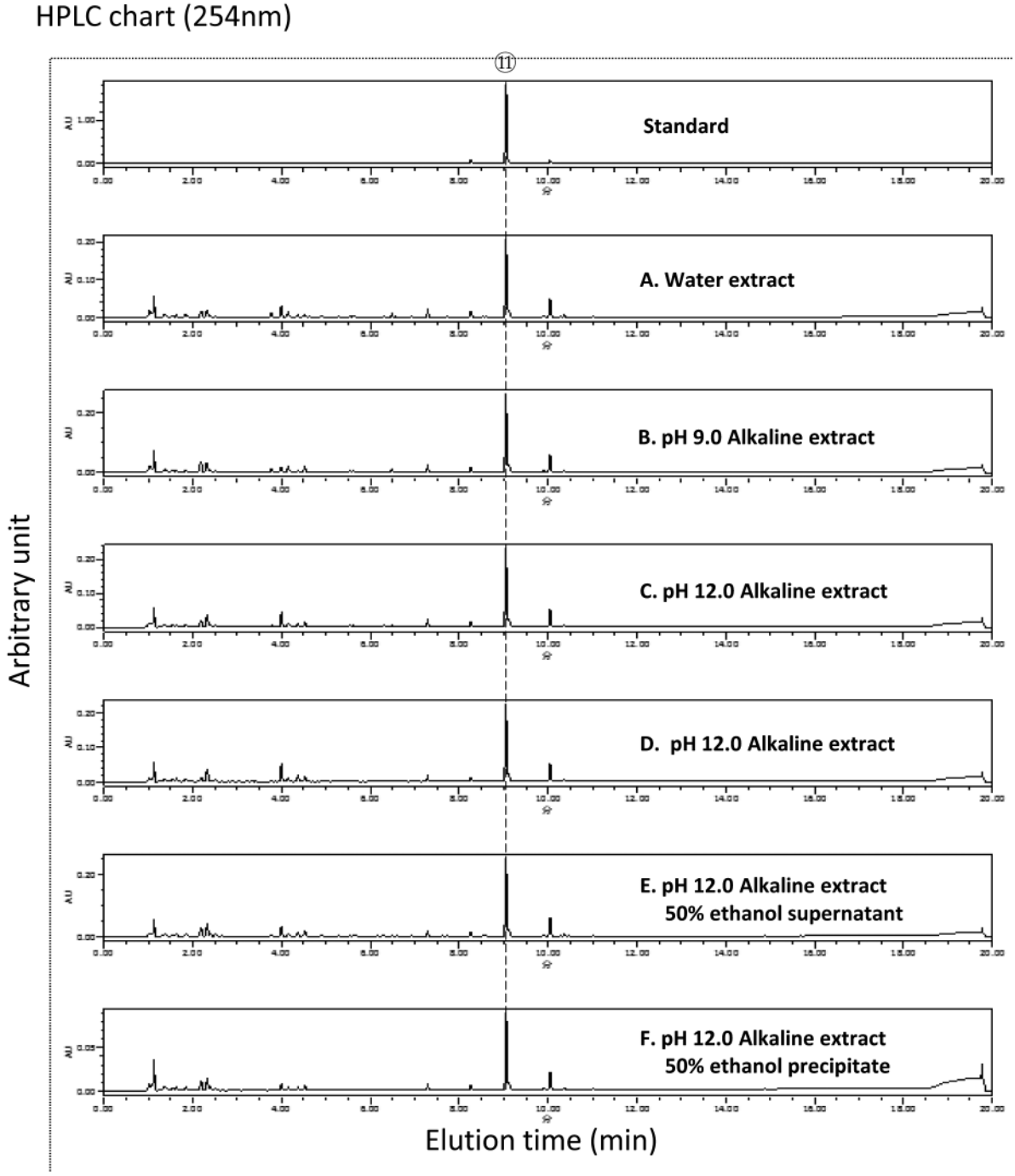

Figure 4. *High performance liquid chromatography separation of standard (11: glycyrrhizic acid.), water extract (A), pH 9.0 alkaline extract (B), pH 12.0 alkaline extract (C), pH 12.0 alkaline extract (D) and 50% ethanol-soluble (E) and -insoluble (F) fractions of D.*

retention times for 6β-hydroxytestosterone and 11α-progesterone were about 4.3 and 6.0 min, respectively. From the dose–response curve, the concentration that inhibited the CYP3A4 activity by 50% (IC_{50}) was determined.

Radical-scavenging activity. The free radical intensity was determined at 25˚C, using electron-spin resonance (ESR) spectroscopy (JEOL JES REIX, X-band, 100 kHz modulation frequency; JEOL Ltd., Tokyo, Japan) (10). The instrument settings

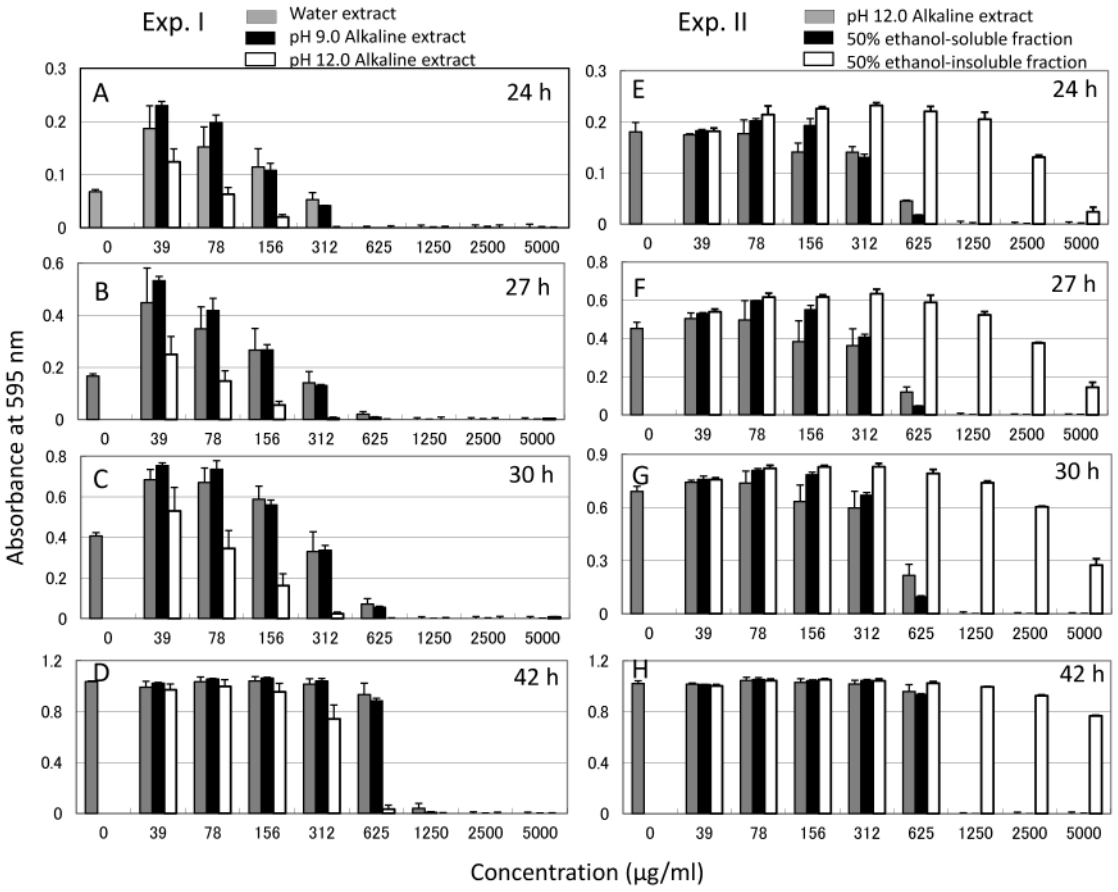

Figure 5. *Antibacterial activity of water and alkaline extracts, and sub-fractions of alkaline extract against Porphyromonas gingivalis 381. P. gingivalis 381 was incubated under anaerobic conditions for 24 (A, E), 27 (B, F), 30 (C, G) or 42 (D, H) h at 37˚C in Gifu Anaerobic Medium containing the indicated concentrations of extract and the absorbance at 595 nm of the bacterial suspension was measured. Each value represents the mean±S.D. of triplicate assays. Significantly different at **p<0.01, *p<0.05 relative to the control (0%).*

were: centre field, 335.5±5.0 mT; microwave power, 16 mW; modulation amplitude, 0.1 mT: gain, 630; time constant, 0.03 s and scanning time, 2 min. For the determination of the superoxide anion (in the form of DMPO-OOH), produced by the HX-XOD reaction (total volume: 200 μl) [2 mM HX in 0.1 M phosphate buffer (PB) (pH 7.4) 50 μl, 1 mM DETAPAC 10 μl, 10% DMPO 30 μl, test sample (in PB) 40 μl, PB 40 μl, XOD (0.5 U/ml in PB) 30 μl], the time constant was changed to 0.03 s (10). For the determination of the hydroxyl radical (in the form of DMPO-OH), produced by the Fenton reaction (200 μl) [1 mM $FeSO_4$ (containing 0.2 mM DETAPAC) 50 μl, 0.1 M PB (pH 7.4) 50 μl, 92 mM DMPO 20 μl, test sample (in H_2O) 50 μl, 1 mM H_2O_2, 30 μl], the gain was changed to 160 (10). The concentration that reduced the radical intensity of DMPO-OOH and DMPO-OH by 50% (IC_{50}) was determined by the dose–response curve of triplicate samples.

Statistical treatment. Experimental values are expressed as the mean±standard deviation (SD). Statistical analysis was performed by using Student's *t*-test. A *p*-value <0.01 and <0.05 was considered to be significant.

Results

Compositional analysis of water and alkaline extracts of licorice root. More than eleven compounds were identified in both water and alkaline extracts of licorice root by UPLC and HPLC (Figures 3 and 4). As expected, glycyrrhizic acid was the most abundant compound in these extracts contained followed by liquiritin apioside and licurazid (structures are shown in Figure 2). The total amounts of these compounds extracted by either of two alkaline solutions (pH 9.0 and pH 12.0) were slightly higher than those extracted by water extract, although there was certain difference in the ratio of each component (Table I). The relative compositions of the two alkaline (pH 12.0) extracts prepared independently were nearly identical, confirming the reproducibility of the separation procedure. The 50% ethanol-insoluble fraction, which represents only 3% of the total alkaline extract, contained much lower amounts of flavones, chalcones and triterpensaponin,

Table I. *Distribution of major ingredients into the water and alkaline extracts and their subfractions of licorice root. Each component was quantified by ultra performance liquid chromatography as described in the Materials and Methods section.*

	Content (%)					
	Method I			Method II		
	Water extraction	Alkaline extraction		Alkaline (pH 12.0) extraction		
		pH 9.0	pH 12.0	Unfract.	50% ethanol-soluble fr.	50% ethanol-insiluble fr.
	(A)	(B)	(C)	(D)	(E)	(F)
Glycyrrhizic acid	8.84	10.91	9.88	9.54	10.61	3.59
Liquiritin apioside	1.52	2.55	2.82	2.83	3.12	1.02
Liquiritigenin 7-apiosylglucoside	0.65	1.36	0.76	0.47	1.08	0.38
Liquiritin	0.38	0.74	0.42	0.64	0.74	0.26
Neoliquiritin	0.1	0.24	0.18	0.17	0.35	0.13
Liquiritigenin	0.16	0.13	0.09	0.1	0.11	0.04
Isoiquiritin apioside	0.93	0.9	0.34	0.32	0.36	0.11
Licurazid	1.81	1.1	2.47	2.89	1.98	0.46
Isoliquiritin	0.08	0.09	0.06	0.05	0.06	0.02
Neoisoliquiritin	0.06	0.05	0.11	0.13	0.11	0.03
Isoliquiritigenin	0.04	0.03	0.03	0.03	0.03	0.01

suggesting the presence of lignin–carbohydrate complex and degradation products. Further experiments are required to test this possibility.

Anti-HIV activity. Alkaline (both pH 9.0 and pH 12.0) extracts had much higher anti-HIV activity (SI>14, and >16, respectively) compared to water extract (SI=4) (Exp. I in Table II). When pH 12.0 alkaline extract was fractionated into 50% ethanol-soluble and -insoluble fractions, most anti-HIV activity was recovered from the 50% ethanol-insoluble fraction (SI=114) (Exp. II in Table II), although its anti-HIV activity was much lower than that of four popular anti-HIV drugs (SI=2048-18920).

Antibacterial activity. All extracts and sub-fractions showed bimodal concentration effects [so-called hormesis (11)] on the growth of *P. gingivalis* 381, with stimulatory effects at lower concentrations and inhibitory effects at higher concentrations (Figure 5). The hormesis was more prominent at the earliest stage of bacterial growth. Maximum hormetic response calculated according to (11) was 174% for water extract, 238% for pH 9.0 alkaline extract, and 82% for pH 12.0 alkaline extract, respectively (Figure 5A). At higher concentration, pH 12.0 alkaline extract most efficiently inhibited bacterial growth, followed by pH 9.0 alkaline extract and water extract (Exp. I in Figure 5). For the pH 12.0 alkaline extract, the 50% ethanol-soluble fraction had much higher antibacterial activity than did the 50% ethanol-insoluble fraction (Exp. II in Figure 5).

CYP3A4-inhibitory activity. Water and alkaline extracts of licorice root inhibited CYP3A4 to comparable extents. The 50% ethanol-insoluble fraction exhibited approximately two-fold lower CYP3A4-inhibitory activity, as compared with the 50% ethanol-soluble fraction (Figure 6).

Radical-scavenging activity. Water and alkaline extracts of licorice root scavenged superoxide (O_2^-) generated by hypoxanthine and xanthine oxidase reaction more effectively than did hydroxyl radical (•OH) generated by Fenton reaction. The 50% ethanol-insoluble fraction exhibited the highest superoxide and hydroxyl radical-scavenging activity.

Discussion

The present study demonstrated for the first time that alkaline extracts of licorice root consistently gave much higher anti-HIV activity compared to water extract. When the pH 12.0 alkaline extract was fractionated with 50% ethanol, the major anti-HIV activity was recovered from the 50% ethanol-insoluble fraction. Although the anti-HIV activity of this fraction (SI=114) was approximately seven-times higher than that of the 50% ethanol-soluble fraction (SI>15) and alkaline extracts of bamboo and tea leaf and orange flower (S=3-40) (3, 5), the yield (0.65%) was only 1/32 that of the 50% ethanol-soluble fraction (20.8%) (Table II). This suggests that the crude alkaline extract may be useful for manufacturing anti-HIV products on a large scale.

The present study demonstrated for the first time that pH 12.0 alkaline extract had three-fold higher antibacterial

Table II. *Biological activities of water and alkaline extracts of licorice root and their subfractions.*

	Anti-HIV activity			Antibacterial activity	CYP3A4 inhibition	Radical scavenging	
						O_2^-	•OH
	CC_{50} (μg/ml)	EC_{50} (μg/ml)	SI	IC_{50} (μg/ml)	IC_{50} (μg/ml)	IC_{50} (μg/ml)	IC_{50} (μg/ml)
Exp. I							
Water extract	749	195	4	424	791	176	1067
Alkaline (pH 9.0) extract	>1000	73	>14	368	>1000	266	905
Alkaline (pH 12.0) extract	>1000	63	>16	131	519	199	1057
Dextran sulfate	>1000	0.09	>10668				
Curdlan sulfate	>1000	0.12	>8235				
Azidothymidine (μM)	229	0.012	19778				
2',3'-Dideoxycytidine μM)	1886	0.55	3431				
Exp. II							
Alkaline (pH 12.0) extract	977	23.4	42	474	203	175	1253
50% ethanol-soluble fraction	>1000	65.3	>15	421	239	252	1362
50% ethanol-insoluble fraction	571	5.01	114	3432	543	128	956
Dextran sulfate	812	0.4	2048				
Curdlan sulfate	>1000	0.14	7318				
Azidothymidine (μM)	230	0.012	18920				
2',3'-Dideoxycytidine μM)	1975	0.62	3174				
Bamboo leaf alkaline extract (SE)[a]			40		403		
Green tea leaf alkaline extract (GT-III)[b]			3	952	387	9.2	101
Oolong tea leaf alkaline extract (OT-III)[b]			13	872	141	8.9	99
Orange flower alkaline extract (OF-III)[b]			>15	1731	>1000	190	698
Grapefruit juice EtOAc Fr.[c]					65		
Gallic acid						0.26	2.7
Sodium ascorbate						6.6	4.5

Cited from [a](3), [b](5), [c](8, 9).

activity against *P. gingivalis* than did the water extract, and most of the antibacterial activity was recovered from the 50% ethanol-soluble fraction. Further study is required to identify the active principle responsible for the antibacterial activity. The by-product of the present study is our finding of hormetic stimulation of bacterial growth by lower concentrations (39-156 μg/ml) of both water and alkaline extract of licorice root (Figure 5). We have reported similar hormetic growth stimulation by alkaline extract of bamboo leaf (7). The biological significance of this phenomenon is unclear, thus needed further study for clarification.

Both water and alkaline extracts dose-dependently inhibited CYP3A4 activity (Figure 6), and the magnitude of inhibition was comparable and similar to that of alkaline extracts of bamboo and tea leaves (Table II). However, it should be noted that CYP3A4-inhibitory activity was much less than that of grape fruit juice. This suggests that alkaline extracts seem likely to be safer compared to grape fruit juice, since the latter is expected to enhance the side-effects of CYP3A4-metabolizable drugs that are administered together (12).

Both water and alkaline extracts scavenged superoxide and hydroxyl radicals to comparable extents, although their radical-scavenging activity was two-orders lower than that of sodium ascorbate (apopular antioxidant) and gallic acid (a component of tannin), and one order lower than that of tea extracts that contain much catechin (Table II). Hydroxyl radical is known to be highly cytotoxic and mutagenic (13, 14), and therefore these extracts may prevent or reduce the incidence of hydroxyl radical-induced adverse effects.

Our recent clinical research demonstrated that alkaline extracts of *Sasa senanensis* Rehder leaf and pine cone of *Pinus parviflora* Sieb et. Zucc significantly improved the conditions of patients infected with lichenoid dysplasia (15) and herpes-simplex virus (16), respectively. It remains to be investigated whether alkaline extract of licorice root have similar effects.

In conclusion, the present study demonstrates that alkaline extracts of licorice root had higher anti-HIV and anti-bacterial activity than the corresponding water extract, whereas CYP3A4-inhibitory and radical-scavenging

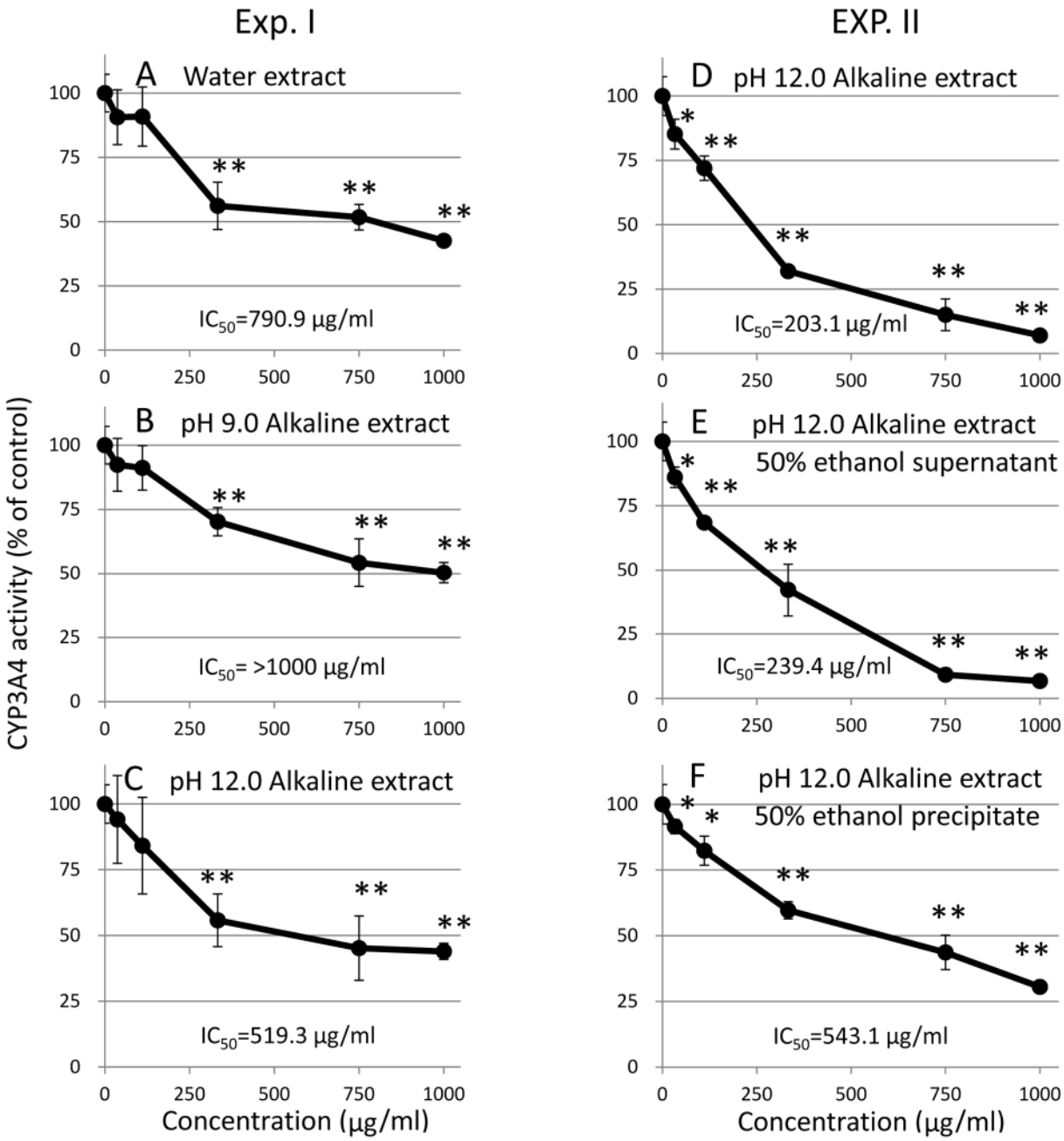

Figure 6. *Cytochrome P450 enzyme (CYP)3A4-inhibitory activity of water extract (A), pH 9.0 alkaline extract (B), pH 12.0 alkaline extract (C) (Exp. I), and 50% ethanol-soluble (E) or –insoluble (F) fraction of alkaline extract (D) (Exp. II). Each value represents the mean±S.D. of triplicate assays. Significantly different at **p<0.01, *p<0.05 relative to the control (0%).*

activity were comparable. Considering that alkaline extraction gave higher amounts of dried materials as compared with water extraction, this method should be considered for efficient utilization of such natural resources.

Conflicts of Interest

This study was funded in part by Maruzen Pharmaceuticals (Hiroshi Sakagami), which caused no prejudice against the impartiality of the research reported.

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