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Synthesis and Reactions of Some New Substituted Benzoxazin-4-One and Quinazolin-4-One

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Abstract. The reaction of 3,4-dichlorobenzoyl chloride with 3,5-dibromo anthranilic acid yielded benzoxazine derivative **(1)**, whose reaction with primary and secondary amines such as benzyl amine, *p*-chloroaniline, *p*-anisidine, *p*-toluidine, piperidine and morpholine in boiling ethanol yielded six (3,5-dibromo-2-(3,4-dichlorobenzoylamino)-N-substituted benzamides **(2-7)**. Reaction of the derivative **(1)** with hydrazine hydrate (1:1 molar proportions) gave the unexpected product 6,8-dibromo-2-(3,4-dichlorophenyl)-3-(2-(3,4-dichlorobenzoylamino)-3,5-dibromobenzamido) quinazolin-4-one **(8)**.

Keywords: benzoxazine derivatives, quinazoline derivatives, 3-thia-1-azabutane-2,4-dione

Introduction

The present investigation deals with synthesis of some new benzoxazine and quinazolinone derivatives bearing a bulky moiety at position -2 in order to study the stability and reactivity of their nucleus towards different nucleophiles. Here we report reactions of 6,8-dibromo-2-(3,4-dichlorophenyl)-*4H*-benzo[d][1,3]oxazin-4-one **(1)** with nitrogen and carbon nucleophiles, aiming to synthesize condensed and non-condensed heterocyclic systems involving quinazoline moiety due to its significant biological activities as anticonvulsant (Dandia *et al*., 2005) as well as antihistamic agents, (Amine *et al.*, 1996), inhibition of cathepsin (Gutschow *et al.*, 2002), besides other antihyperglycemic activities (Ram *et al.*, 2003) and in continuation of other investigations directed towards the synthesis and reaction of some benzoxazine and quinazoline derivatives (Ma *et al*., 2006; Zheng *et al.*, 2006).

Materials and Methods

Melting points are uncorrected. IR spectra were recorded on a Pay-Unicam SP3-2000 spectrophotometer using KBr wafer technique. The ^{1}H-NMR spectra 200 MHz were determined on a Varian Gemini using TMS as internal reference (chemical shifts are expressed as δ, ppm). Micro-analytical data (C, H, N) were obtained from the Microanalytical Center at Cairo University. The physical data are listed in Table 1.

6,8-Dibromo-2-(3,4-dichlorophenyl)-*4H*-benzo [d] [1,3] oxazin-4-one (1). To a solution of 3,5-dibromoanthranilic acid (0.01mol) in dry 50 ml pyridine, 3,4-dichlorobenzoyl chloride was added dropwise with stirring.The reaction mixture was heated on water bath for 2 h, and then poured onto acidified cold water, The separated solid was filtered off, dried and crystallized from benzene to give compound **1** as yellow crystals. IR: 1773 cm^{-1} (C=O lactone), 1622 cm^{-1} (C=N) and 1600 cm^{-1} (C=C); ^{1}H NMR (DMSO): δ 7.63-7.90 (m, 3H) and 8.07-8.45 (m, 2H).

3,5-Dibromo-2-(3,4-dichlorobenzoylamino)-N–substituted benzamides (2-7). A mixture of **1** (4.49 g, 0.01 mol) and primary amines and/or secondary amines namely benzyl amine, *p*-chlroaniline, *p*-anisidine, *p*-toluidine, piperidine and morpholine (0.01 mol) in 50 ml ethanol was refluxed for 3 h. The solid obtained, while heating and after concentration of solvent was filtered off and recrystallized from a suitable solvent to give compounds **2-7**.

3,5-Dibromo-2-(3,4-dichlorobenzoylamino)-N-benzyl benzamide (2). IR: 3270 cm^{-1} (NH) and 1650 cm^{-1} (C=O); ^{1}H NMR (DMSO): δ 4.52(m, 2H), 7.18-7.22 (m, 5H phenyl ring), 7.72-8.13(m, 5H halogenated rings), 8.93-8.97(s, 1H) and 10.38-10.40(s, 1H).

3,5-Dibromo-2-(3,4-dichlorobenzoylamino)-N-(4-chlorophenyl)benzamide (3). IR:3250 cm^{-1} (NH) and 1660 cm^{-1} (C=O).

3,5-Dibromo-2-(3,4-dichlorobenzoylamino)-N-(4-methoxyphenyl) benzamide (4). IR:3240 cm^{-1} (NH) and 1660 cm^{-1} (C=O).

3,5-Dibromo-2-(3,4-dichlorobenzoylamino)-N-(4-methylphenyl) benzamide (5). IR:3260 cm^{-1} (NH) and 1650 cm^{-1} (C=O).

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Table 1. Characterization and physical data of synthesized compounds

Compound nos.	M.p.(°C)	Solvent (yield%)	M.F (M. Wt.)	Calc.% (found%) C	H	N
1	187-188	B	$C_{14}H_5Br_2Cl_2NO_2$	37.37	1.12	3.11
		80	449.91	36.96	1.97	2.98
2	269-270	D	$C_{21}H_{14}Br_2Cl_2N_2O_2$	45.27	2.53	5.03
		70	557.06	45.21	2.51	4.90
3	281-282	B/EtOH	$C_{20}H_{11}Br_2Cl_3N_2O_2$	41.59	1.92	4.85
		80	577.48	41.27	1.73	4.84
4	271-272	B/EtOH	$C_{21}H_{14}Br_2Cl_2N_2O_3$	44.01	2.46	4.88
		80	573.06	43.86	2.34	4.80
5	234-235	D	$C_{21}H_{14}Br_2Cl_2N_2O_2$	45.27	2.53	5.03
		80	557.06	45.94	2.81	4.96
6	130-132	Pet. (80/100)/B	$C_{19}H_{16}Br_2Cl_2N_2O_2$	42.65	3.01	5.23
		70	535.05	42.34	2.87	5.08
7	252-254	D	$C_{18}H_{14}Br_2Cl_2N_2O_3$	40.25	2.63	5.22
		70	533.87	40.72	2.09	4.96
8	254-256	D	$C_{28}H_{12}Br_4Cl_4N_4O_3$	36.80	1.32	6.13
		90	913.85	36.33	1.23	5.98
9	Over300	DMF	$C_{14}H_6Br_2Cl_2N_2O_2$	36.16	1.30	6.02
		60	464.92	37.56	1.28	5.56
10	230-232	D	$C_{14}H_7Br_2Cl_2N_3O$	35.53	1.59	11.05
		70	463.94	35.80	1.30	10.07
11	250-252	D	$C_{15}H_8Br_2Cl_2N_4O_2$	36.24	1.52	9.06
		80	506.96	36.09	1.42	8.97
12	191-192	D	$C_{29}H_{12}Br_4Cl_4N_4O_4S$	35.76	1.24	5.75
		60	973.92	34.98	1.21	5.72
13	211-212	B/EtOH	$C_{17}H_7Br_2Cl_2NO_5$	38.09	1.32	2.61
		40	535.95	38.12	1.51	2.59
14	220-221	B/EtOH	$C_{14}H_7Br_2Cl_2NO_3$	35.93	1.51	2.99
		50	467.92	35.67	1.55	2.92
15	Over300	DMF	$C_{14}H_7Br_2Cl_2N_2O$	37.45	1.35	6.25
		80	448.93	37.34	1.24	6.00
16	191-192	Pet. (80/100)/B	$C1_{14}H6Br_2C_{12}N4O_2$	34.11	1.22	11.36
		70	492.94	33.98	1.12	11.23

EtOH =ethanol; B = benzene; D = 1,4-dioxane; DMF = N,N-dimethylformamide; Pet. = petroleum ether

3,4-Dichloro-N-(2,4-dibromo-6-(piperidine-1-carbonyl) phenyl)benzamide (6). IR: 3230 cm^{-1} (NH) and 1660 cm^{-1} (C=O).

3,4-Dichloro-N-(2,4-dibromo-6-(morpholine-4-carbonyl)phenyl) benzamide (7). IR:3240 cm^{-1} (NH) and 1680 cm^{-1} (C=O).

6,8-Dibromo-2-(3,4-dichlorophenyl)-3-(2-(3,4-dichloro-benzoylamino)-3,5-dibromobenzamido)quinazolin-4-one (8). A mixture of compound **1** (4.49 g,0.01 mol) and hydrazine hydrate (0.5 g, 0.01mol) in 50 ml. ethanol was refluxed for one h. The solid that separated while refluxing was filtered off and recrystallized from 1,4-dioxane to give compound **8** as white crystals. IR: 3300-3500 cm^{-1} (NH) or enolic (OH), 1645 cm^{-1} (CO); ^{1}H NMR (DMSO): δ 8.50 (d, 1H), 8.45 (d, 1H), 8.36 (s, 1H), 7.91 (m, 4H), and 5.71 (br, 1H, NH).

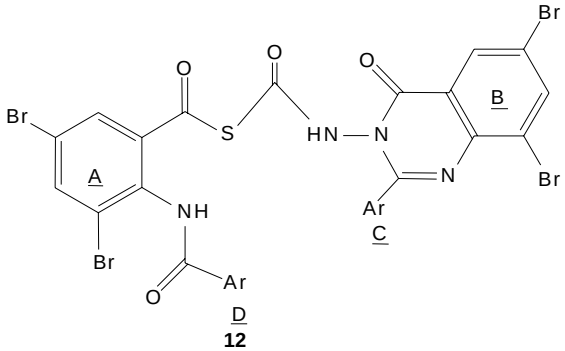
6,8-Dibromo-2-(3,4-dichlorophenyl)-3-N-substituted quinazolin-4-one (9,10, 11). A mixture of compound **1** (4.49 g, 0.01 mol) and primary amines, namely, hydrazine hydrate, hydroxylamine hydrochloride and/or semicarbazide hydrochloride (0.01 mol) was refluxed in 50 ml *n*-butanol for 4 h. The solid that formed while refluxing was filtered off and recrystallized from a suitable solvent to give comounds **9, 10,** and **11**.

6,8-Dibromo-2-(3,4-dichlorophenyl)-3-hydroxyquinazoline-4-(3H)-one (9). IR: 3440 cm^{-1} (NH), 1684cm^{-1} (CO).

3-Amino-6,8-dibromo-2-(3,4-dichlorophenyl)quinazoline-4-(3H)-one (10). IR: 3200 cm^{-1} (NH), 3450-3315 cm^{-1} (NH_2) and 1688 cm^{-1} (C=O).

1-(6,8-Dibromo-2-(3,4-dichlorophenyl)-4-oxoquinazolin-3-(4H)-yl)urea (11). IR: 3211 cm^{-1} (NH), 3330-3260 cm^{-1} (NH_2) and 1672 cm^{-1} (C=O).

1-(6,8-Dibromo-2-(3,4-dichlorophenyl)quinazolin-4-one-3-yl)-4-(2-(3,4-dichlorobenzoylamino)-3,5-dibromophenyl)-3-thia-1-azabutane-2,4-dione (12). A mixture of compound **1** (4.49 g, 0.01 mol) and thiosemicarbazide (0.91 g, 0.01 mol) in 50 ml ethanol was refluxed for 4 h. The solid that separated was filtered off and recrystallized from 1, 4-dioxane to give compound **12** as colourless crystals. IR: 3220-3370 cm^{-1} (NH/OH) 1685 cm^{-1} (CO) and 1610-1590 cm^{-1} C=N/C=C); ^{1}H NMR (DMSO): δ 7.65 (s, 1H, CONH), 7.84-8.05 (m, 4 H aromatic A & B), 8.22-8.39 (m, 3H aromatic C), 8.062-8.063 (m, 3H aromatic D) and 10.45 (s, NH exchangeable with D_2O).



12

2-(6,8-Dibromo-2-(3,4-dichlorophenyl)-*4H*-benzo [d] [1,3] oxazin-4-ylidene) malonic acid (13) and 3,5-Dibromo-2-(3,4-dichlorobenzamido) benzoic acid (14). A mixture of compound **1** (4.49 g, 0.01 mol) and active methylene compounds namely, ethyl cyanoacetate and /or diethyl malonate (0.01 mol) in 10 ml. dry pyridine was refluxed for 10 h. The reaction mixture was poured onto crushed ice and acidified with 10% HCl, 20 ml; the precipitate was filtered off, washed with water and dried. Products **13** and **14** were separated by fractional crystallization using benzene and ethyl alcohol, respectively.

2-(6, 8-Dibromo-2-(3, 4-dichlorophenyl)-4 H-benzo [d] [1,3] oxazin-4-ylidene) malonic acid (13). IR: 1610cm^{-1}(CN) 1700 cm^{-1} (CO) and 3350-3500 cm^{-1} (OH); ^{1}H NMR (DMSO): δ 7.93-8.08 (m, 3H aromatic 8.29-8.34 (m, 2H aromatic) and 10.55(s, 2H, 2COOH)].

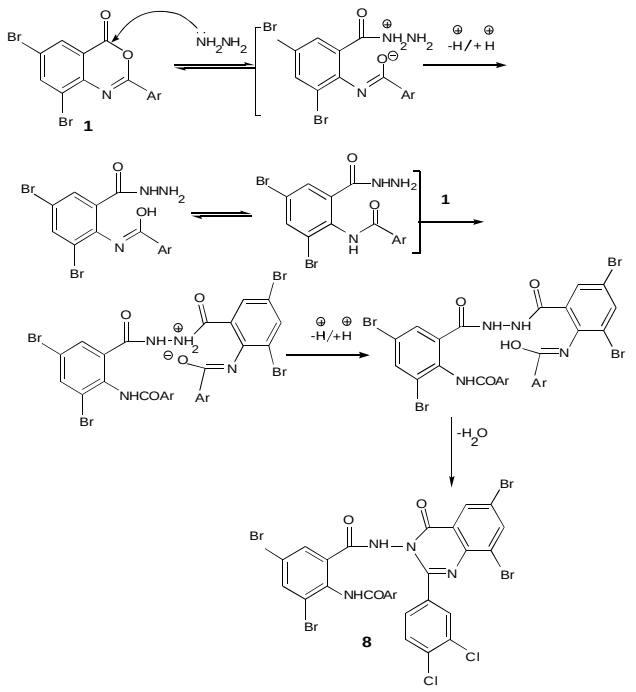
3,5-Dibromo-2-(3,4-dichlorobenzamido) benzoic acid (14). IR: 1680 cm^{-1} (amido CO) 1690 cm^{-1} (CO), 3270 cm^{-1}(NH) 3570 cm^{-1} (OH); ^{1}H NMR (DMSO): δ 7.92-8.08 (m, 3H aromatic) 8.28-8.34 (m, 2H aromatic) and 10.56 (*s*, 2H, COOH, NHCO).

6,8-Dibromo-2-(3, 4-dichlorophenyl)quinazoline-4*(3H)*-one (15). A mixture of compound **1** (4.49 g, 0.01 mol) and 20 ml formamide and/or amm. acetate (0.73 g, 0.01 mol) was fused on oil bath at 190 °C for an h. The reaction mixture was poured onto cold water and the solid formed was filtered off, washed with water, dried and recrystallized from *N*, *N* dimethlyformamide to give compound **15** as pale yellow crystals. IR:1684 cm^{-1} (CO), 3445 cm^{-1} (NH), 1610 cm^{-1} (C=N); ^{1}H NMR (DMSO): δ 13.12 (s, 1H NH or OH, exchangeable with D_2O) and 7.92-8.34 (m, 5H, aromatic).

3,5-Dibromo-2-(5-(3,4-dichlorophenyl)-*1H*-tetrazol-1-yl) benzoic acid (16). A mixture of compound **1** (4.49 g, 0.01 mol) and sod. azide (0.65 g, 0.02 mol) in acetic acid (20 ml) was refluxed for 12 h. The solvent was removed and the residue was washed with water (3x20 ml), filtered off, dried and crystallized from pet. ether (80-100)/benzene mixture to give compound **16** as light yellow crystals. IR:3445cm^{-1} (OH) and 1690 cm^{-1} of (CO); ^{1}H NMR (DMSO): δ 11.1 (s, 1H COOH, exchangeable with D_2O) and 7.92-8.30 (m, 5H aromatic).

Results and Discussion

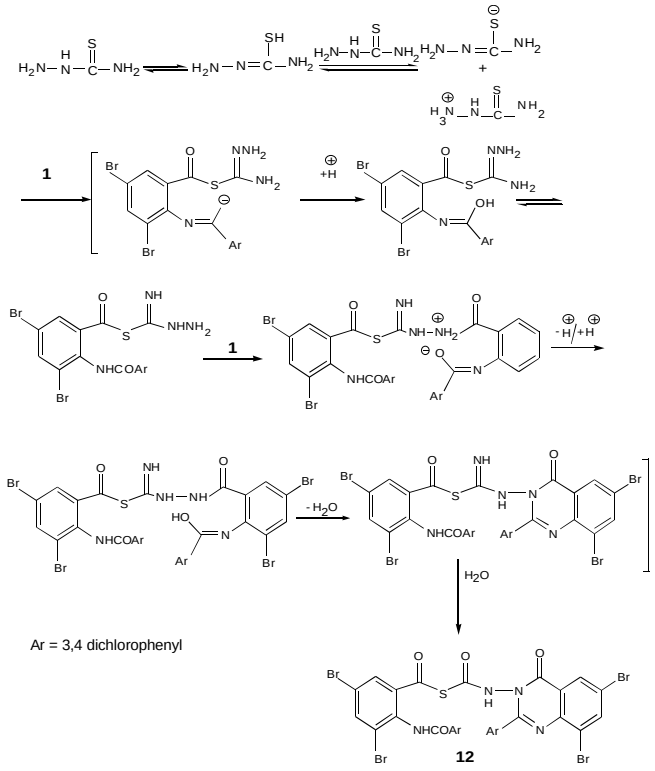
The benzoxazine derivative **(1)** was prepared in situ by the reaction of 3,4-dichlorobenzoyl chloride with 3,5-dibromo anthranilic acid. Reaction of compound **1** with primary and secondary amines such as benzyl amine, *p*-chloroaniline, *p*-anisidine, *p*-toluidine, piperidine and morpholine in boiling ethanol yielded (3,5-dibromo-2-(3,4-dichlorobenzoylamino)-N–substituted benzamides (compounds **2-7**) (scheme 1). Also reaction of compound **1** with hydrazine hydrate (1:1 molar proportion) gave the unexpected product 6,8-dibromo-2-(3,4-dichlorophenyl)-3-(2-(3,4-dichlorobenzoylamino)-3,5-dibromobenzamido)quinazolin-4-one (**8**). Formation of compound **8** take place probably according to the following mechanism:

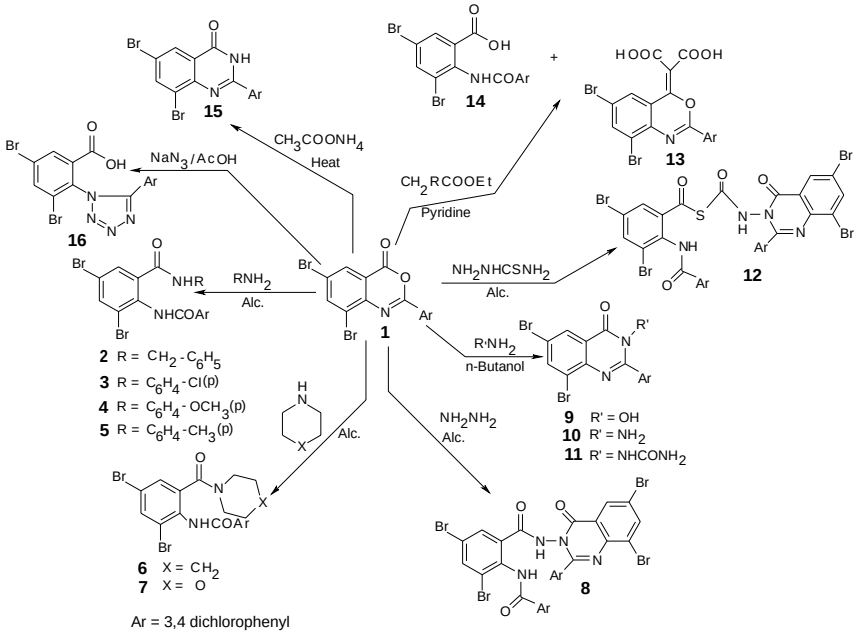


On the other hand, reaction of compound **1** with hydrazine hydrate, hydroxylamine hydrochloride and/or semicarbazide hydrochloride in boiling *n*-butanol gave 6, 8-dibromo 2-(3′,4′-dichlorophenyl)quinazolin-4-one derivatives. (**9,10,11**).

According to our interests in developing new condensed and non condensed heterocyclic systems, (El-Ziaty and Shiba, 2007), compound **1** was treated with thiosemicarbazide in boiling ethanol giving 1-(6,8-dibromo-2-(3, 4-dichlorophenyl) quinazolin-4-one-3-yl)-4-(2-(3,4-dichlorobenzoylamino)-3,5-dibromophenyl)-3-thia-1-azabutane-2,4-dione **(12)** as unexpected product in contrary to that previously reported, by Nassar and Aly (2002) and Mohamed *et al.* (1981) (scheme 1).

Formation of compound **12** probably takes place according to the following mechanism:

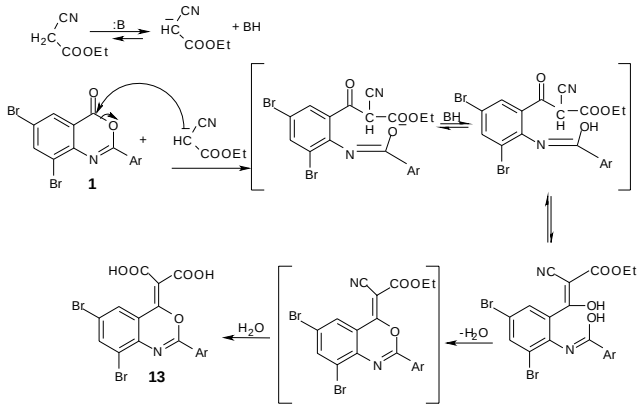




Scheme 1

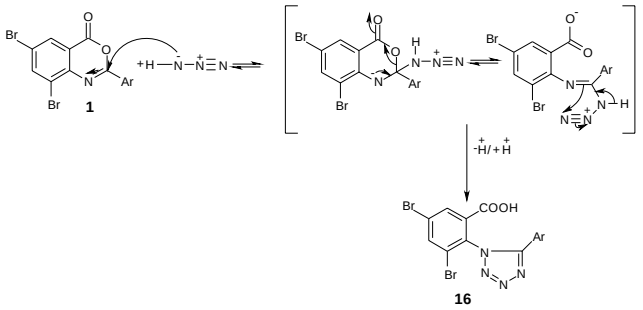
By studying reaction of compound **1** with active methylene compounds, namely, ethyl cyanoacetate and/or diethyl malonate in pyridine afforded 2-(6,8-dibromo-2-(dichlorophenyl)-4*H*-benzo[*d*][1,3]oxazin-4-ylidene)malonic acid **(13)** and 3,5-dibromo-2-(3,4-dichlorobenzamido)benzoic acid **(14),** the open form of **1**, respectively.

Formation of compound **13** probably takes place according to the following mechanism:



Fusion of compound **1** with ammonium acetate and/or formamide yielded the corresponding 6,8-dibromo-2-(3,4-dichlorophenyl) quinazolin-4-(*3H*)-one **(15)** as previously reported (Nassar and Aly, 2002). Ring opening of **1** with hydrazoic acid gave 3,5-dibromo-2-(5-(3,4-dichlorophenyl)-*1H*-tetrazol-1-yl) benzoic acid **(16)** (scheme 1).

Formation of compound **16** probably takes place according to the following mechanism:



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Synthesis of Some 2-Methyl-3-(Arylthiocarbamido) Quinazol-4-Ones and 2-Methyl-3-(Arylidencarboxamido) Quinazol-4-Ones as Potential Antimicrobial Agents

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Abstract. Some quinazolone derivatives of 2-methyl-3-(arylthiocarbamido) quinazol-4-ones **(2)** and 2-methyl-3-(arylidencarboxamido) quinazol-4-ones **(3)** have been synthesized and assayed for their possible antibacterial activity against *Bacillus subtilis, Bacillus cereus, Salmonella aureus, Salmonella lutae* and antiviral activity against *Gomphrena mosaic* virus. Some of these compounds show notable activity.

Keywords: quinazol-4-ones, antibacterial activity, antiviral activity

Introduction

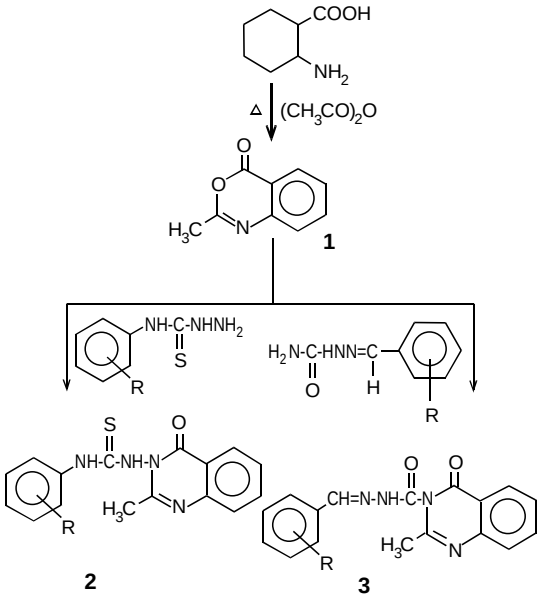
Quinazolone derivatives exhibit a wide range of activity such as dopamine receptor (Srivastva *et al.,* 1987) anthelmintic (Gupta *et al.,* 1988; Alaimo, 1972) anti-inflammatory (Alagarsamy *et al.,* 2003), antimicrobial (Pandey *et al.,* 2004; Alagarsamy *et al.,* 2000) CNS deprcssant (Saksena and Khan, 1989; Kacker and Zaheer, 1951) neuroleptic (Mukerji *et al.,* 1980) hypotonic (Gujral *et al.,* 1955) and analgesic (Ram *et al.,* 1990). Pharmacological activity of this class of compounds is beyond any doubt, thus it was decided to synthesize some new title quinazolones in order to study their antibacterial and antiviral activities.

Materials and Methods

Melting points were determined in open glass capillary and are uncorrected. IR spectra (ν_{max} in cm^{-1}) were recorded on a Perkin Elmer-157 spectrometer and ^{1}HNMR (60 MHz) spectra on Varian EM 360 spectrometer.

2-Methyl-1,3-benzo [d] oxazin-4-one called acetanthranil was obtained essentially by the method of Zentmyer and Wagner (1949).

2-Methyl-3-(4-chlorophenylthiocarbamido)-quinazol-4-one (**2**, R=4-CI). (Scheme I) Acetanthranil (1.6 g) and 4-chlorophenylthiosemicarbazide (1.8 g) in methanol (20 ml) were heated together upto 3 h. The reaction mixture was cooled. The solid thus obtained was washed with dil. Na_2CO_3 followed by dil. HCl and the product was finally crystallised from ethanol; yield 75%. m.p. 168°C; MS: m/z: M^+ 344; IR(KBr) cm^{-1} : 1150, 1595, 1570, 1440, (Ar-H), 1620 (C=N), 1660 (C=O), 3250 (NH); ^{1}HNMR (DMSO-d_6): δ 2.1 (s, 3H. CH_3), 7.0-7.5 (m, 8H, Ar-H), 8.2 (s, 2H, NH); Anal. found: C 55.61; H 3.64; N 16.12. ca1cd: C 55.73; H 3.79; N 16.25% (Table 1).



Scheme 1

2-Methyl-3-(4-methoxybenzylidencarboxamido) quinazol-4-one (**3**, R=4-OCH_3). Acetanthranil (1.6 g) and 4-methoxybenzaldehydesemicarbazone (1.7 g) in presence of excess of acetic anhydride (10 ml) were heated together on a water bath for 3 h. The reaction mixture was cooled. The solid thus obtained was washed with dil. Na_2CO_3 followed by dil. HCI and the product was finally crystallised from ethanol; yield 79%, m.p. 166 °C; MS m/z : M^+ 336; IR (KBr) cm^{-1}: 1595, 1570, 1460 (Ar-H), 1650 (C-NH), 1670 (C=O), 3400 (NH); ^{1}HNMR (DMSO-d_6): δ 2.1 (s, 3H, CH_3), 4.2 (s, 3H, OCH_3), 6.1 (s, 1H, N=CH), 7.2-7.6 (m, 8H, ArH), 8.6 (s, 1H, NH);

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Table 1. Characterization data of compounds **2** and **3**

Compound	R	M.P. °C	Yield (%)	M. F.	Found (calcd. %) C	H	N
2a	H	140	72	$C_{16}H_{14}N_4OS$	61.8	4.39	17.86
					(61.91	4.54	18.04)
2b	2-CH_3	120	78	$C_{17}H_{16}N_4OS$	62.83	4.83	17.03
					(62.94	4.96	17.27)
2c	3-CH_3	149	72	$C_{17}H_{16}N_4OS$	62.84	4.82	17.01
					(62.94	4.96	17.27)
2d	4-CH_3	178	75	$C_{17}H_{16}N_4OS$	62.82	4.81	17.02
					(62.94	4.96	17.27)
2e	4-OCH_3	164	70	$C_{17}H_{16}N_4O_2S$	59.87	4.6	16.31
					(59.99	4.73	16.45)
2f	2-Cl	210	77	$C_{16}H_{13}N_4OSCl$	55.62	3.63	16.13
					(55.73	3.79	16.25)
2g	4-Cl	168	75	$C_{16}H_{13}N_4OSCl$	55.62	3.63	16.13
					(55.73	3.79	16.25)
2h	2,4-Cl_2	162	75	$C_{16}H_{12}N_4OSCl_2$	50.55	3.1	14.62
					(50.67	3.18	14.77)
2i	2,6-$(CH_3)_2$	175	72	$C_{18}H_{18}N_4OS$	63.8	5.22	16.39
					(63.88	5.36	16.55)
3a	H	148	70	$C_{17}H_{14}N_4O_2$	66.56	4.46	18.24
					(66.67	4.6	18.28)
3b	4-CH_3	203	78	$C_{18}H_{16}N_4O_2$	67.38	4.88	17.35
					(67.49	5.03	17.48)
3c	4-OCH_3	166	79	$C_{18}H_{16}N_4O_3$	64.2	4.64	16.52
3d	2-Cl	194	80	$C_{17}H_{13}N_4O_2Cl$	59.8	3.71	16.29
					(59.92	3.84	16.44)
3e	4-Cl	191	77	$C_{17}H_{13}N_4O_2Cl$	59.89	3.7	16.28
					(59.92	3.84	16.44)
3f	2-OH	211	72	$C_{17}H_{14}N_4O_3$	63.22	4.23	17.23
					(63.35	4.37	17.38)
3g	2-OH, 3-OCH_3	297	74	$C_{18}H_{16}N_4O_4$	61.24	4.4	15.78
					(61.36	4.57	15.90)

Anal. found: C 64.20; H 4.64; N 16.52. calcd: C 64.28; H 4.79; N 16.66% (Table 1).

Pharmacology. ***Antibacterial screening.*** The *in vitro* antibacterial activity of the synthesised compounds was determined by the method of Verma and Nobbles (1968), at a concentration of 100 µmg/ml. A standard tetracycline was also tested under similar conditions to compare the results. The inhibition zone (in cm) against four species *viz.*: *B. subtilis*, *B. cereus*, *S.aureus* and *S. lutes* were measured. The results are recorded in Table 2.

Antiviral screening. The *in vitro* antiviral activity of all the synthesized compounds reported here was determined by the method of Verma and Awasthi (1978) on *Gomphrena mosaic* virus, taking gaur leaves as host. The concentration of each sample was 3.0 mg/mole. The percentage activity is recorded in Table 2.

Results and Discussion

Antibacterial activity. All the compounds of this report have been screened for their antibacterial activity. Perusal of the

Table 2. Antibacterial and antiviral activities of compounds **2** and **3**

Compound	R	Inhibition zone (in cm)				% inhibition of
		a	b	c	d	*Gomphrena mosaic* virus
2a	H	1.3	-	1.1	1.3	40
2b	2-CH_3	1.5	1	1.4	1.7	52
2c	3-CH_3	2	0.8	0.9	2.1	48
2d	4-CH_3	1.8	2.2	1.6	1.7	52
2e	4-OCH_3	2.6	-	0.9	0.8	22
2f	2-Cl	1.7	1.1	1.5	1.6	60
2g	4-Cl	2.8	2.2	2.7	2	65
2h	2,4-Cl_2	2.4	2	2.9	2	77
2i	2,6-$(CH_3)_2$	2.6	-	0.8	1.1	20
3a	H	0.6	0.6	0.8	1.1	24
3b	4-CH_3	1.2	0.8	0.9	1	50
3c	4-OCH_3	-	-	1.5	0.7	15
3d	2-Cl	-	1.7	1.2	1.6	18
3e	4-Cl	2.1	-	0.8	1.8	27
3f	4-OH	1.8	1.6	1.5	1.3	61
3g	4-OH, 3-OCH_3	2.2	1.6	1.9	1.9	80
Tetracycline*		3.2	2.4	2.4	2.4	-

a = *B. subtilis*; b = *B. cereus*; c = *S. aureus*; d = *S. lutae;** = standard drug for antibacterial activity; - = no activity.

results (Table 2) indicates that the range of inhibition zones produced by these compounds are 0.6 to 2.8 cm on *Bacillus subtilis;* 0.6 to 2.2 cm on *Bacillus cereus;* 0.6 to 2.9 cm on *Salmonella aureus* and 0.7 to 2.1 cm on *Salmonella lutes*, respectively, whereas, the standard drug (tetracycline) could inhibit the bacteria in the zones of 3.2, 2.4, 2.4, 2.4, respectively. Compounds **2g**, **2h**, **3e** and **3g** showed good activity on all the four species as compared to the standard drug. So further screening of these compounds on wider range of bacteria as well as on more dilutions is desirable. On the basis of above, the following conclusion can be drawn:

(i) The presence of -OCH_3 group in the phenyl ring does not impart any activity.
(ii) Presence of alkyl group (-CH_3) in phenyl ring works better than unsubstituted phenyl ring.
(iii) The position of -CH_3 group in the phenyl ring played a role in the activity. The order of activity is 4-CH_3 > 2-CH_3 > 3-CH_3.
(iv) The presence of one -Cl group played notable activity while two chlorine atoms together work better.
(v) Presence of -OH group alone or in presence of -OCH_3 group imparts better antibacterial activity.

Antiviral activity. All the compounds have been screened for their antiviral activity against *Gomphrena mosaic* virus. The results (Table 2) indicate that the activity was between 15 to 80%. Compounds **2f**, **2g**, **3f** and **3g** have activities greater than 60%, while two compounds **2h** and **3g** had activities 77% and 80%, respectively. Further screening of these two compounds on wider range of viruses as well as more dilutions is in progress. These results lead to the following conclusion.

(i) Presence of -Cl and -OH groups in phenyl ring alone and presence of -OH with -OCH_3 group increases activity.
(ii) As the number of Cl-atom increases, the activity also increases.
(iii) Presence of phenolic -OH at position-4 in the phenyl ring imparts much more activity than if it is at position-2.

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Some Physical Characteristics and Nutritional Composition of the Seeds of Wild Pepper (*Erythrococca anomala,* Benth)

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Abstract. Study of physical properties and nutritional components of whole and powdered wild pepper seeds (*Erythrococca anomala,*Benth) revealed that the seeds have good parameters for machineability. The contents of moisture, ash, protein, lipid and carbohydrate and major and trace minerals were found in functional quantities, while the heavy metals were negligible or absent. Thus the seeds are potential source of nutrients and can be used as additive in food product development.

Keywords: *Erythrococca anomala,* nutritional composition, product development, seed machineability

Introduction

Seeds are abundantly found in nature and are good and cheap sources of foods. They have multiple nutritive values and are also known to contain reasonable quantities of edible oils and fats. The satiety value, flavour enhancing and hunger delaying abilities are the particular attributes of fats. Moreover, seeds are cheap source of protein, known to be very important for the normal body functions in animals. Considering the shortage of food nutrients in human diet and reliance of the country on imports of food products from foreign countries, lot of efforts have been focussed on the exploitation of locally available natural raw materials for food production. For example, work has been done on bitter kola (*Garcinia kola*) (Daramola and Adegoke, 2007), African oil bean seed (*Pentaclethra macrophylla*) (Ajibola, 2005), African breadfruit seeds (*Treculia africana*) (Omobuwajo, 2002) etc.

Wild pepper (*Erythrococca anomala,* Benth) is an indigenous plant whose seeds are popular among the local people in the Western part of Nigeria, owing to the benefits associated with them. Its seeds are locally known as Iyere (Yor), Monsoro (Hausa) and Osunrisa (Ghana). They are usually of reddish to yellow colour on ripening, while deep brown after drying.

The plants are creepers, found clustering around the stems of cocoa and kolanut trees, proliferating freely in parts of Southern Nigera. The seeds of *E.anomala* are aromatic, pungent and medicinally used in treatment of sore throat, mouth infections, preparation of herbal soups for women and new mothers etc. The seeds are also used as food additive (in flavouring 'kunnusaki', preparation of pepper soups, cooking of rice and meat). Recently, the seeds of *E. anomala* have been employed in perfumeries in the northern Nigeria.

Since, no work has been rendered on determining the physical characteristics and the nutritional potential of the seeds, the present study was undertaken to establish the attributes of the seeds of wild pepper (*E. anomala,* Benth).

Materials and Methods

The physical characteristic including sphericity index, aspect ratio and density (kernel density, bulk density and density ratio) were determined using the methods of Maduako and Faborode (1990) and Mohsenin (1986).

Properly dried seeds of *E. anomala* were milled using Shromadzu grinding machine (AGG -270 F 005028F4) and sieved using a 6 mm mesh size. The powdered seeds were then made into 50 packs and kept for further analysis.

The moisture content was determined by drying method, protein by Kjedhal digestion method, and crude fat by Soxhlet extraction, according to AOAC (1990). The ash content was determined using Toyo Seisokusho Muffle Furnace (KL420: 00004023021) at 550 °C for one h.

Minerals analysis. Use of atomic absorption spectrophotometer, AAS-324-75603-84 & 2P88887GM, was made for the minerals analysis.

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Samples were prepared by ashing the weighed amount of powdered seeds in a Seisokusho Muffle Furnace PK50067FT; 4022004) at 500 °C which was then allowed to cool. The ashed sample was digested and diluted serially with 1N HCL. The digest was filtered and aspirated into the AAS, where the minerals were automatically quantified.

Results and Discussion

The sphericity index (56.70 ± 0.001) and the aspect ratio (87.00 ± 0.031%) of the seeds of *E. anomala* (Table 1) were found to lie within the range predicted for objects with round shapes (Akande, 1998). The values obtained equally falls within the range predicted by Omobuwajo (2002), for African breadfruit seeds (*Treculia africana*) which have the appearance similar to that of *E. anomala*.

The density characteristics of grains predict their machine-ability. The density of seeds was proportional to the material components in the given food sample which is according to the throught-put capacity of the machine (loading through the hopper to the action zone of the machine) and in relation to the power of the machine (Romeo, 2000). The seed kernel density was 1.01 x 10 ± 0.017 kg/m^3 , bulk density was 0.59 x 10 ± 0.002 kg/m^3 and the density ratio was 58% (Table 1). These values are within the range of values obtained for the seeds of a similar grain, African breadfruit seeds (*Treculia africana*) which predicts adequate value for grain density characteristics in relation to the material components of the seed (Akande, 1998).

Table 1. Physical characteristics of the seeds of *E. anomala*

Parameters	Values
Sphericity index (%)	56.70 ± 0.001
Aspect ratio (%)	87.00 ± 0.31
Mean kernel density (kg/m^3)	1.01×10 ± 0.017
Bulk density (kg/m^3)	0.59×10 ± 0.002
Density ratio (%)	58%

The nutritional composition of food materials is crucial in the study of foods. Specific nutritional values of the food are recorded for various applications in food processing, food preservation, product development and engineering applications (Gordon, 1990). The result of the proximate analysis is shown in Table 2.

Although the proximate composition of the seeds of *E. anomala* has not been officially documented, Adebisi (2006), who studied the effects of various drying temperatures on the seeds, quote the base values close to the values obtained in this study. In addition, these values indicate that the seeds are of adequate nutritional value as recorded by Tull (1985).

The moisture content (Table 2) of 10.6 ± 0.031% of the seeds is less than 12% (moisture content predicted for grain storeability), thus the seeds can actually have a long shelf-life when needed to be stored before utilization or processing (Ihekoronye and Ngoddy, 1985).

Table 2. Proximate composition of seeds of *E. anomala*

Nutrients	Percentage composition
Moisture content	10.60 ± 0.311
Ash content	4.95 ± 0.095
Protein content	15.12 ± 0.033
Lipid/fat content	11.33 ± 0.069
Carbohydrates	58.00

The ash contents of 4.95 ± 0.095% is approximately equal to 5% which is believed to be adequate enough to contain reasonable quantities of minerals i.e., predisposing the seeds to be rich in minerals.

The lipid content of 11.33 ± 0.069, shows that the seeds cannot be categorized as oil seeds; an oil seed should contain at least 40% or above lipids. However, it proves that the seeds contain some non-polar compounds that are phytochemicals, essential oils and essential fatty acids.

The protein and carbohydrate contents were 15.12 ± 0.033% and 58.00%, respectively. These values show that the seeds are rich in carbohydrates but protein content is at a lower level. The protein value of 15.12 ± 0.033 is actually not enough to fulfil the daily dietary requirement of an average body weight of 70 kg, however, when the seed is used as a part of food product, it can contribute meaningfully to protein intake. Carbohydrates are known to supply energy to the body and equally act as “protein sparer” so that proteins can be spared to perform their primary function. Tull (1985) stated that carbohydrates should be used in preference to proteins as energy supplier so that proteins can be used for body growth and repairs. Since the seeds of *E. anomala* contain higher percentage of carbohydrates than proteins, proteins are spared to perform their primary function when used in product development.

The mineral elements determined in this study (Table 3) include major elements, required in amounts greater than 100 mg/day, trace elements required in less than 100 g/day and the heavy metals that are not required at all. Mineral elements are chemicals required by the body for optimal physiological activities and are required in small quantities by the body

systems. However, since they cannot be synthesized by the body, they are to be supplied in the diet or food consumed (Ihekoronye, 1987). The values obtained for the minerals present in the seeds of *E. anomala* especially the major ones (Mg, Fe, Ca, Na) are appreciable and so can support the daily dietary requirements. The trace mineral elements Mn, P, K, and Zn were found in functional amounts while the heavy metals were negligible or not found at all (Table 3). The implication of this is that the seed is a potential source of the essential macro and micro mineral elements needed by human body.

Table 3. Mineral composition of the seeds of *E. anomala*

Minerals	Trials 1	2	3	Mean ± SD (µg/mg)
K	0.4479	0.4339	0.4393	0.44 ± 0.0076
Ca	1.3295	1.3132	1.2746	1.306 ± 0.028
Mg	3.2703	2.8722	3.2109	3.118 ± 0.215
Zn	3.3583	3.3279	3.3460	3.344 ± 0.015
Fe	3.3800	3.0545	3.0943	3.1760 ± 0.178
Mn	0.8422	0.8534	0.8353	0.844 ± 0.009
Na	0.9240	0.9250	0.9211	0.9233 ± 0.016
Al	nil	nil	nil	nil
P	0.0460	0.0445	0.0450	0.0452 ± 0.006
Pb	nil	nil	nil	nil
Co	nil	nil	nil	nil

Conclusion

The physical characteristics and nutritional qualities of the seeds of *Erythrococca anomala* Benth were determined. The seed dimensions and the shape were established as being spherical. The seeds contain appreciable quantities of macro-nutrients (proteins, fats and carbohydrates) and mineral elements. It can, therefore, be concluded that the seeds of *E.anomala* can be mechanically processed and used in food product development.

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Relative Study of the Colour Fastness of Cotton, Woolen and Silk Fabrics Dyed With Walnut Bark Dye

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Abstract. Natural walnut dye was extracted from walnut bark and applied to cotton, woolen and silk fabrics with the same depth of colour and colour fastness was assessed. Walnut dye had good saturation on all the three fabrics and its colour fastness properties ranged between good and excellent.

Keywords: walnut bark dye, *Juglan regia*, natural dye, colour fastness

Introduction

Natural and synthetic dyes are used for dyeing of fabrics and pottery. Some synthetic dyes such as disperse dyes and azo amine dyes have harmful effects on human beings causing allergy, cancer etc., and are anti-environment (IARC, 1975; Scott, 1952). Natural dyes are less allergenic, non-toxic and environment friendly and can be used in textile, pharmaceutical, food and cosmetic industry safely (Ali *et al.*, 2007). Shades produced with most of the natural dyes are not bright, so mordants are used to produce fast and bright colours (Gulrajani and Gupta, 1992), while some dyes are substantive and can be directly applied on the textile fabrics, wool and leathers, without any need of mordants.

The drawback associated with natural dyes is that there are no suitable standard shade cards and standard test procedures relating to their extraction and other dyeing properties. A lot of work is, therefore in progress to improve poor reproducibility and lack of desirable properties of natural dyes (Ali *et al.*, 2007).

The present work is concerned with the extraction of natural dye from the *Juglan regia* (walnut), dyeing of various fabrics (cotton, woolen and silk) with it and then studying the fastness properties.

Juglan regia (walnut) belongs to the family Junglandaceae. It is a slow growing tree in northern parts of Pakistan. It is planted mainly for timber and nuts. The husk is smooth and nuts are easy to split (Cannon and Cannon, 1994). Green hulls or rinds of walnut were used for dyeing. The roots, inner bark referred to as walnut bark, was also used even though it had less potency of colour than the rind (Rita, 1971). Fruit is excellent for eating and baking. It is often used in confectionery and ice cream.

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All parts of the tree especially bark and nuts contain a substantive brown dye. This dye can be used to give various shades of brown and yellow. The colours are fast and permanent. Mordant may be used to produce a range of shades particularly with chrome, copper, alum and iron etc. The bark dye gives a pure colour to wool by applying bismuth and tin as mordant and brown violet on long simmering (Cannon and Cannon, 1994).

The most important dye pigment in walnut is Juglone, which is a derivative of naphthoquinones. Purified Juglone gives a red orange dye, which can be modified by the presence of tannins and flavonoids in the plant. Biologically active naphthoquinones are secondary metabolities of many plants. Juglone (5-hydroxy-1,4-naphthaquinone) is a naturally occurring naphoquinone that forms derivatives which are more extractable substances of the roots, leaves and green skin of walnut. Juglone and its derivatives have a wide spectrum of applications in folk medicine, cosmetics, pharmaceuticals and agro-eco system protection. Naphthoquinone derivatives have been used as antiviral and antifungal constituent of man preparation for skin colouring and hair colour dyes (Tomaszkiewicz and Vogt, 2004).

Materials and Methods

Instruments. D400 IR dyeing machine (SDL Atlas England); Launderometer (Roaches), Perspirometer kit (SDL Atlas England); oven, Ci 3000 + Xenon; weatherometer (Atlas England); water bath; grey scales for staining (ISO 105 A03); grey scale for change in shade (ISO 105 A02); crockmeter (SDL Atlas England); multifiber (DW).

Chemicals. Detergent ECE (without optical brightener), sodium per borate, l-histidine monochloride monohydrate, sodium dihydrogen orthophosphate, distilled water, sodium

carbonate, sodium hydroxide, acetic acid, sulphuric acid, and perchloroethylene solvent.

All the chemicals and solvents used were of AR grade.

Collection of bark and extraction of colour. The most often used parts of plant for dyeing are leaves and fruit husk (Onal *et al.,* 2004). In this study only bark was used for extraction of the dye.

Walnut bark was purchased from Murree market and thoroughly washed with water and dried. It was ground into powder and sieved through 22 mesh size strainer. 500 g bark powder was soaked in 5 litre water overnight, boiled for 2 to 3 h and then subjected to stirring for 3 to 4 h at simmering temperature. A dark brown coloured dye solution obtained was filtered and kept for dyeing and other tests (Kongkachuichay *et al.,* 2002).

Dyeing with walnut dye. 20 g Fabrics of cotton, wool and silk each were dyed with the same depth of walnut bark dye extract in the D400 IR dyeing machine (SDL Atlas England) with programmes to control temperature (100 °C), time 1 h and speed of circulation 1.5 rpm. The three dyed fabrics were used to study the colour fastness to washing, perspiration, rubbing fastness, light fastness, dry cleaning, fastness to water and sea water, heat fastness and spotting to alkaline and acid colour fastness (Paul *et al.,* 2003).

Fastness determination. Wash fastness test of all the three dyed fabrics was determined according to ISO 105 C06 method. Light fastness was examined according to ISO 105 standard method procedure B02. Rubbing fastness (dry and wet) test was carried out according to ISO 105x12 standard test procedure. Colour fastness tests to dry cleaning, water, sea water, and perspiration (acidic and basic) were carried out according to ISO 105: D01, E01, E02 and E04 methods, respectively. Colour fastness to spotting of acids and alkalies tests were performed according to ISO E05 and E06 methods, respectively (BS 1006: 1990).

Washing fastness: Washing fastness was determined by preparing the soap solution containing 4 g detergent and 1 g sodium perborate per litre of distilled water. Then pH was adjusted to 10.5±0.1 by addition of approx. 1 g of sodium carbonate. Cotton, woolen and silk fabric pieces of size. 10 x 4 cm were attached to multifiber DW of the same measurements by sewing along with one of the shorter sides. The three composite specimens were put into glasses of launderometer (Roaches) for 30 min at 60 °C having liquor ratio 50:1. Launderometer or Washtec consists of a water bath containing a rotatable shaft which supports radially, stainless steel container (75±5 mm diameter x 125±10 mm height) of capacity 550±50 ml, the bottom of the container being 45±10 from the centre of the shaft. The shaft/container assembly is rotated at a frequency of 40±2/min. After 30 min, samples were removed from the Washtec. Stitches were removed and the specimens were dried at temperature not more than 60 °C. The change in stain and in shade was assessed with the help of grey scale.

Colour fastness to perspiration. Tests were carried out by dipping the fabrics into l-histidine monohydrochloride monohydrate solution according to ISO 105 E04 method. Specimens of cotton, wool and silk of 4 cm x 10 cm measurement were attached to pieces of multifibre of the same measurement by sewing alongwith one of shorter sides and dipped separately into alkaline and acidic solutions for 30 min having liquor ratio 50:1. Then the cotton, wool and silk specimens were placed in the perspirometer kits and the desired pressure was applied. Perspirometer kits are test devices each consisting of a frame of stainless steel into which a weight piece of mass 5 kg and base of 60 mm x 115 mm is closely fitted so that a pressure of 12.5 kpa can be applied on test specimens measuring 40 mm x 100 mm, placed between glass or acrylic resin plates measuring 60 mm x 115 mm x 1.5 mm. The test device is constructed in such a way that a pressure of 12.5 kpa remains unchanged. The perspirometer kits (acidic and basic) for tests of the three fabrics were placed in the vacuum oven for 4 h and then the kits were removed from the oven and the stitches were opened except on one shorter side. Specimens were dried at 60 °C by hanging in air. Change in the colour of each specimen and staining of the adjacent fabric (DW) were assessed with grey scale.

Rubbing fastness. Dry rubbing on cotton was carried out with the help of crockmeter under a pressure of 9N in to and fro movements on standard rubbing cloth. Test sample cotton of 5 cm x 14 cm measurement was taken. Both warp and weft readings were noted. Same procedure was adopted for wool and silk and values were taken with the help of grey scale.

Wet rubbing. Wet rubbing on cotton fabric was done under the same conditions of crockmeter as in the dry rubbing except the standard rubbing cloth was soaked into 100% deionized water. Same procedure was repeated with woolen and silk fabrics and the change in colour and in stain was assessed with the help of grey scale.

Light fastness. Light fastness was carried out according to ISO 105 standard procedure B02; in weatherometer by Atlas. Xenon arc lamp was used which is an artificial light source representative of natural day light D65. Fabrics of measurement 7 cm x12 cm of cotton, wool and silk were exposed to

Xenon arc lamp for 24 h, at standard testing conditions using blue wool as standard reference fabric. The above three treated fabrics were compared with grey scale for evaluation.

Colour fastness to dry cleaning. Undyed cotton twill bags of 10 cm x 10 cm measurement were stitched around three sides and cotton, woolen and silk pieces of 4 cm x 10 cm measurement were placed into separate bags alongwith 12 non-corrodable steel disks and the fourth side of the bag was sewed. Then the bags were placed in separate containers of Washtec containing 200 ml of perchloroethylene solvent and agitated for 30 min at 30±2 °C. Afterwards the bags were removed from the container. The samples were squeezed to remove surplus solvent and dried in the air by hanging them at a temperature of 60±5 °C. Assessment of change in colour of samples and change in colour of solvent was carried out with the help of grey scale.

Colour fastness to water and sea water. Colour fastness to water and sea water was evaluated in the same manner as for the colour fastness to perspiration. ISO-105 E01 and E02 methods were used for water and sea water, respectively. In case of water, fabrics were dipped in deionized water, while for colour fastness to sea water, fabrics alongwith multifibers were dipped in NaCl solution (30 g/l) for 30 min. For both water and sea water the above three treated composite fabrics were put in perspirometer kit. These kits were placed in the oven for 4 h at 37±2 °C. Then the specimens were dried at temperature not more than 60 °C. Change in shade and in stain were noted with the help of grey scale.

Colour fastness to dry heat. Dry hot pressing was done according to ISO 105 XII. Specimens of cotton, wool and silk were pressed at temp. 110±2 °C with hand iron and change in colour was assessed with grey scale.

Colour fastness to spotting acids and alkali. Spots of acetic acid 300 g/l, sulphuric acid 50 g/l, tartaric acid 100 g/l and Na_2CO_3 100 g/l of water were put on the specimens and change in shade was assessed with ISO-105 A02 grey scale.

Results and Discussion

Change in staining. When the washing fastness properties of cotton, woolen and silk fabrics were compared, it was observed that on diacetate band of multifiber DW for cotton fabric, grey scale gave good (4-5) rating, while for woolen and silk fabrics, staining was excellent (5). For cotton band of multifiber, staining was good for both woolen and silk fabrics (4-5) rating and (4) for cotton fabric. For nylon band of multifiber, cotton fabric gave satisfactory (3-4) results, woolen gave (4) rating and silk gave good (4-5) rating. For polyester band all the fabrics gave the same rating of 4. For polyacrylic and wool bands, all the three fabric gave excellent (5) rating for staining (Table 1).

Change in shade. Results for change in shade gave satisfactory (3-4) rating for cotton, (4) for woolen and (2-3) rating for silk which is poor as compared to the other two fabrics. Results for change in shade for cotton, woolen and silk dyed with walnut bark extract are given in Table 1.

Colour fastness to acidic and basic perspiration. Results of acidic and basic perspiration can also be seen in Table 1.

Table 1. Washing fastness, alkaline and acidic perspiration fastness of fabrics

Fabric	Washing fastness rating						
	Diacetate	Cotton	Nylon	Polyester	Polyacrylic	Wool	Change in shade
Cotton	4-5	4	4	5	5	5	3-4
Wool	5	4-5	4	5	5	5	4
Silk	5	4-5	4	5	5	5	2-3
Fabric	Basic perspiration						
Cotton	4-5	4	4	5	4-5	4-5	4-5
Wool	5	4-5	4	5	5	5	4-5
Silk	5	4-5	4-5	5	5	4-5	5
Fabric	Acidic perspiration						
Cotton	4-5	4	4	5	5	4-5	4
Wool	5	4-5	4	5	5	4-5	4-5
Silk	4-5	5	4-5	5	4-5	4-5	4-5

***Cotton fabric*.** On diacetate band of cotton fabric the results of acidic and basic perspirations were good (4-5). For cotton and nylon band acidic and basic perspiration results for staining were 4. For polyester band both basic and acidic perspiration gave excellent (5) results. For polyacrylic band it was found excellent (5) for acidic perspiration and good (4-5) for basic perspiration. For wool band, results for both perspirations were good (4-5). Change in shade for acidic perspiration was 4, while it was good (4-5) for basic perspiration for cotton fabric.

***Woolen fabric*.** Acidic and basic perspiration gave excellent (5) rating for diacetate bands of multifiber. For cotton bands change in stain was good (4-5) for acidic and basic perspirations. For nylon bands staining was same i.e. (4). For polyester and polyacrylic bands staining for both perspiration were excellent (5). Wool band also gave excellent staining (5) for basic perspiration and good (4-5) for acidic perspiration; change in shade were good (4-5) for both acidic and basic perspiration.

***Silk fabric*.** For silk fabric dyed with walnut dye, diacetate band showed excellent (5) rating for basic perspiration and good (4-5) rating for acidic perspiration. For cotton bands of multifiber, change in stain was good (4-5) for basic perspiration and excellent (5) for acidic perspiration. For polyester band rating for staining was excellent (5) for both acidic and basic perspiration. For polyacrylic band it was excellent (5) for basic perspiration and good (4-5) for acidic perspiration for silk fabric. For nylon band basic and acidic perspiration gave good (4-5) rating results on silk. Wool band also gave good (4-5) rating for silk fabric for acidic and basic perspiration. Change in shade gave excellent rating (5) for basic perspiration and good rating (4-5) for acidic perspiration.

Light fastness. Results of light fastness are shown in Table 2 for cotton, wool and silk dyed with walnut bark dye extract. Cotton and silk gave rating (4) while wool gave good rating (4-5).

Dry cleaning. Results of change in colour were excellent (5) for cotton, woolen and silk fabrics. Change in colour of solvents was also excellent (5) for all the three fabrics.

Rubbing fastness: *Dry rubbing fastness*. Cotton and wool both gave good rating (4-5) for dry rubbing fastness along warp and weft whereas the silk fabric showed excellent rating (5) for dry rubbing fastness along warp and weft.

***Wet rubbing fastness*.** For cotton fabric, wet rubbing along warp was 3-4 which was satisfactory and acceptable. Along weft wet rubbing was 4. For woolen fabric wet rubbing fastness along warp and weft was 3-4, which is also acceptable. Silk fabric gave wet rubbing fastness rating good (4-5) along warp and weft. Results are shown in Table 2.

Table 2. Rubbing fastness, light fastness and colour fastness to dry cleaning

Fabric	Rubbing fastness				Light fastness	Colour fastness to dry cleaning	
	dry rubbing		wet rubbing		change in shade of fabric	change in shade of fabric	change in shade of solvent
	warp	weft	warp	weft			
Cotton	4-5	4-5	3-4	4	4	5	5
Wool	4-5	4-5	3-4	3-4	4-5	5	5
Silk	5	5	4-5	4-5	4	5	5

Colour fastness to water: *Change in stain*. For cotton fabric diacetate band gave good (4-5) rating. For woolen and silk fabric the rating of change in stain were excellent (5). For cotton band of multifiber DW all three fabrics showed same rating i.e. 4-5 which was good. For nylon band, cotton and silk gave rating 4 for staining while woolen gave good (4-5) rating for staining. For polyester band results were excellent (5) for all the three fabrics. Polyacrylic band showed good rating (4-5) for cotton and silk and excellent rating (5) for wool. Wool band of multifiber gave rating of 4 for staining of cotton, 4-5 rating for woolen and rating 5 for silk fabric (Table 3).

***Change in shade*.** Rating for change of colour fastness to water for all the three fabrics was good (4-5). Results are shown in Table 3.

Colour fastness to sea water. Results of colour fastness to sea water are also given in Table 3.

Staining. For diacetate band cotton fabric showed rating of 4, wool showed excellent (5) rating and silk showed good (4-5) rating for staining. Cotton band of multifiber showed excellent (5) rating for staining of cotton, wool and silk with walnut bark dye. Nylon band gave good (4-5) results for wool and silk and excellent (5) rating for cotton fabric. Polyester band gave good rating for staining (4-5) for cotton and excellent rating (5) for both wool and silk. Polyacrylic band showed excellent staining (5) for both wool and silk and good (4-5) rating for cotton fabric. Wool band of multifiber (DW) gave 5, 4-5 and 4 stain rating for cotton, wool and silk, respectively (Table 3).

Table 3. Results of colour fastness to water and sea water, dry heat fastness and spotting to acids and alkali

	Colour fastness to water						
Fabric	Diacetate	Cotton	Nylon	Polyester	Polyacrylic	Wool	Change in shade
Cotton	4-5	4-5	4	5	4-5	4	4-5
Woolen	5	4-5	4-5	5	5	4-5	4-5
Silk	5	4-5	4	5	4-5	5	4-5
	Colour fastness to sea water						
Fabric	Diacetate	Cotton	Nylon	Polyester	Polyacrylic	Wool	Change in shade
Cotton	4	5	5	4-5	4-5	5	4-5
Woolen	5	5	4-5	5	5	4-5	4-5
Silk	4-5	5	4-5	5	5	5	4-5

	Colour fastness to spotting			
Fabric	Dry heat fastness (at 110 °C)	Acid spotting		Alkali spotting Na_2CO_3 (100 g/l)
		Acetic acid (300 g/l)	Sulphuric acid (150 g/l)	
Cotton	4-5	4-5	4	3-4
Wool	4-5	4-5	3-4	3
Silk	4-5	4-5	4	4

Change in shade for sea water. Change in shade was good (4-5) for all the three fabrics dyed with walnut bark dye extract for sea water (Table 3).

Dry heat fastness: At 110 °C cotton, woolen and silk fabrics gave good (4-5) rating during dry hot pressing. Results are given in Table 3.

Colour fastness to acidic and basic spotting. Change in shade for cotton, woolen and silk fabrics, on spotting with acetic acid (300 g/l) gave good rating (4-5). Sulphuric acid (150 g/l) spotting gave rating 4 for cotton and silk and satisfactory (3-4) rating for wool. Alkali spotting (100 g/l Na_2CO_3) gave satisfactory rating (3-4) for cotton and 3 for wool, while rating of 4 for silk fabric. Results are tabulated in Table 3.

Conclusion

Results showed that natural walnut bark dye has excellent to good colour fastness properties. Colours are fast and permanent. Dye can be applied without mordant and has good saturation on cotton, woolen and silk fabrics. It is environment friendly and has no health hazard effects.

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Quantitation of Fatty Acids by GLC and Separation of Omega-6 Nutraceutical Fatty Acid From *Carthamus tinctorius* L. Seed Oil Cultivated in Pakistan

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Abstract. The GLC analysis of *Carthamus tinctorius* (safflower) yielded average hexane extracted oil content of 28% (25-30%); the oil contained high level of linoleic acid (74%). Monounsaturated fatty acid, oleic acid amounted 12.94%, while the saturated fatty acids like palmitic acid and stearic acid were 9.43 and 1.81%, respectively. Iodine value of linoleic acid was found to be 160.1 while its purity was 93.1%.

Keywords: *Carthamus tinctorius*, linoleic acid, omega-6 fatty acids

Introduction

Carthamus tinctorius (safflower) is an annual species of the family compositae. This crop is adapted to dry land or irrigated cropping system. It is also known as false saffron, thistle saffron, cartame, saffron baturd in English, while Arab calls it Kazhirah (Eckey and Miller, 1954; George, 1892) and in Pakistan (Sindh) it is commonly known as Kushumba, Khusakdana or Powariji bij (Jafri, 1966). Seeds usually mature in September, about four weeks after the end of flowering (Oelke *et al*., 1989) with a seed oil content between 30-45%.

The plant is cultivated in California, Middle East, Africa and India (Eckey and Miller, 1954). It is an indigenous crop in Pakistan and is cultivated in Gilgit, Hunza, Kotri and Mirpur (Jafri, 1966). Normally two varieties of *C. tinctorius*, are found, one that produces oil with high amount of monounsaturated fatty acid (oleic acid) and the other with high concentration of polyunsaturated fatty acid (linoleic acid). *C. tinctorius* is a valuable source of one of the most important nutraceutical fatty acid, omega-6 (linoleic acid), an essential fatty acid which cannot be synthesized by the body but plays an important role in the control of joint ailments, skin, hair, nail and scalp disorders like eczema, acne, psoriasis etc.

The present study was made on the seeds of *C. tinctorius* variety producing linoleic acid and growing in Pakistan. The aim of the study was to assess the fatty acid composition of *C. tinctorius* in order to separate the linoleic acid quantitatively using urea adduct separation technique. This procedure is frequently applied to obtaina polyunsaturated or branched chain fatty acids in concentrated form. Toxicity studies were also carried out to check the feasibility of using the oil as a source of essential fatty acid. Such studies based on the application of urea complexation technique have not yet been reported by any author in Pakistan.

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Materials and Methods

All the reagents (analytical and GC) used, were purchased from E-Merck/Sigma/Aldrich. Pure standards of fatty acid methyl esters were obtained from Supelco Chemicals Co.

Extraction of oil. Seeds (one kg) were taken from the seed cultivars, cleaned to remove admixtures then grinded in an electrical grinder and fed to a soxhlet extractor fitted with a 2 litre round bottom flask and a condenser. The extraction was done on a water bath for 4-6 h with 1.5 litre *n*-hexane. Then the solvent was distilled off under vacuum in rotary evaporator. The oil was dried over anhydrous sodium sulphate, filtered and weighed. The procedure was performed in triplicate.

Fatty acid composition. Fatty acid methyl esters were prepared according to the standard IUPAC method (IUPAC, 1987) and analyzed on a Perkin-Elmer gas chromatograph model Clarus 500, fitted with a polar capillary column SP 2340 (60 m x 0.25 mm) and a flame ionization detector. Oxygen free nitrogen was used as a carrier gas at a flow rate of 3.5 ml/min.

Other operational conditions were as follows: initial oven temperature 70 °C for 5 min, increase in temp @ 10 °C/min to 180 °C and then @ 3 °C/min to 220 °C, held for 8 min; FID temperature: 275 °C, injector: 250 °C.

A sample volume of 1.0 µl was injected and the total analysis time was 37 min. Fatty acid methyl esters were identified by

comparing their relative retention time to those of authentic standard of fatty acid methyl esters obtained from Supelco Co. All of the quantification was done by a built-in data handling programme provided by the manufacturer of gas chromatograph. The data was transferred to HP Laser Jet-1300 printer attached to the instrument. The fatty acid composition was reported as a relative percentage of the total peak area.

Toxicity studies of *C. tinctorius* oil. The prescribed daily dose of the test material was 1-2 table spoon (5-10 ml) twice a day for an adult person. To ensure safe evaluation, the test group animals (albino rats) were administered 5 ml/kg body weight of the test drug. Olive oil was purchased from the local market in a sealed tin pack and used as standard reference oil for comparative study; it was also given in a dose of 5 ml/kg of body weight.

Before proceeding to toxicity studies, animals (males and females) were housed separately and kept under keen observation for a period of 20 days with free access to food and water. Any animal showing sluggish movement or any sign of illness was not included in the test.

Acute oral toxicity studies. Acute oral toxicity study was conducted in albino rats. The rats were divided into three groups, each group comprising of 10 animals (5 male and 5 female). Test material was administered in a single dose by means of a gavage to group A i.e., test group, according to the standard method of acute oral toxicity test (Loomis, 1978). Group B was given standard branded olive oil and group C was the control. Observations with reference to physicobehavioural changes and mortality within 24 h were made. Animals were further observed for a period of 72 h for changes in behavioural pattern and delayed mortalities.

Separation of omega-6 (linoleic) fatty acid from the oil. *C. tinctorius* oil was converted to its methyl ester by its methanolysis in presence of sodium as catalyst. The mixed fatty acid methyl esters were fractionated by urea adduct formation. The procedure was adopted as follows:

C. tinctorius oil (100 gm) was dried on steam bath, at controlled temperature of 80 °C. The mixed fatty acid methyl esters were prepared by transesterifiction process (IUPAC, 1987).

35 g Urea was added to a solution of 50 g of mixed ester in 300 ml methanol and dissolved while heating. The solution was kept overnight at -5 °C then filtered off. The precipitate was again washed with methanol, cooled to -5 °C and combined with the washed liquid and filtrate. Again 30 g urea was added to the filtrate and the mass was warmed until the urea was completely dissolved. The solvent was cooled and kept at 5 °C overnight; the crystallizate was filtered off and washed with 200 ml cold methanol. The washing and filtrate were combined and finally 20 g urea was added and the above mentioned procedure was again repeated. The last filtrate was concentrated to obtain linoleate. Pure linoleate was weighed to calculate the percentage recovery. The experiment was performed in triplicate.

Purity of the product was determined by iodine value and compared with the theoretical values, mentioned in the literature.

Results and Discussion

Oil content. The triplicate analysis of *C. tinctorius* seeds yielded oil content in the range of 25-30% with an average yield of 28%. This value complies with the values earlier reported by others (Hamrouni *et al*., 2004; Eckey and Miller, 1954). The oil content of *C. tinctorius* is comparatively higher than that of the other most commonly used oil seeds such as soybean seeds containing 20% oil (Eckey and Miller, 1954). Hence, in this consideration, *C. tinctorius* seeds may be used for commercial purposes.

Fatty acid composition. The fatty acid composition of oil from *C. tinctorius* seeds is summarized in Table 1; chromatogram is shown in Fig. 1. The oil contained fatty acids, generally present in seed oils such as saturated fatty acids including palmitic and stearic acids and unsaturated fatty acids including oleic, linoleic and linolenic acids. Average values are resultant of three analysis on GC. *C. tinctorius* was found to be an excellent source of pure linoleic acid (Parker *et al*., 1955) which is normally considered as one of the most important essential nutraceutical fatty acids of biomedical importance.

Oleic acid , 12.41%, found in the oil, is in close agreement with the earlier reported values (Swern, 1964) while the present value of omega-6 (linoleic acid) fatty acid of 74%, is also in good conformance with Gecgel *et al*. (2007) and Swern (1964). It means that the dependence of the fatty acid composition of *C. tinctorius* on the location of the plant is comparatively little. Total saturated acids were found to be 11.24%. Fatty acid composition determined in the present work is also in good agreement with the conventional *C.tinctorius* fatty acid profile reported by Knowles (1989).

Table 1. Fatty acid composition of *C. tinctorius* oil (av. wt %)

Palmitic acid C_{16}	Stearic acid $C_{18.0}$	Oleic acid $C_{18.1}$	Linoleic acid $C_{18.2}$	Linolenic acid $C_{18.3}$
9.43	1.81	12.94	73.98	0.93

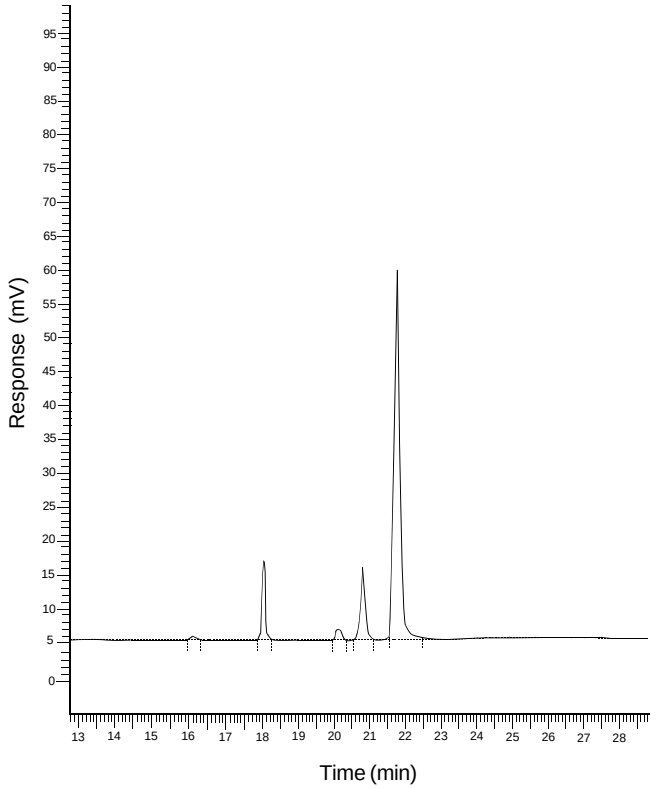

Fig. 1. Chromatogramme of *C. tinctorius* seed oil.

Toxicity studies. Oral administration of safflower oil for 42 days in a dose of 5.0 ml/kg body weight was not found to produce any toxic effect in both male and female animals in comparison with the standard and the control group (Table 2).

Table 2. Acute oral toxicity studies

Group	Av. wt. of animals (kg)	Dosage	Results
A	150-200	5 ml / kg body wt. (safflower oil)	- no mortality - all animals found normal in their activities - there was no sign of any untoward effects during said period of observation.
B	150-200	5 ml / kg body wt. (olive oil)	same as above
C	150-200	control (placebo)	same as above

Preparation of linoleic acid. The technique of separation of polyunsaturated fatty acid by urea adduct complexation was first described by Bengen (1940). The main findings of Bengen was that urea can be used to separate straight chain compounds from branched or cyclic compounds; later, numerous investigators confirmed this method. The new technique was used for the preparation of methyl oleate (Swern and Parker, 1952; Schlenk and Holman, 1950), for the preparation of concentrates of linoleic acid from safflower oil (Kim *et al*., 2003; Swern and Parker, 1953), for separation of docosahexaenoic acid from algal oil via urea complexation (Senanayak and Shahidi, 2000), gamma linolenic acid concentrate from borage oil (Spurvey and Shahidi, 2000) and preparation of omega-3 PUFA concentrate from fish oil via urea complexation (Ratnayake *et al*., 1988).

The reviews of Schlenk (1953) gives detailed explanation of the application of this technique. In the present findings about 21.7% linoleic acid (omega-6) was separated at the end of experiments performed in triplicate. Low yield of the inclusion compound was also observed earlier by Schlenk and Holman (1950), suggesting that the more highly unsaturated fatty acids may be even more difficult to bind as inclusion compounds.

The purity of linoleate in the present study was found to be 93.1%. The percentage yield, 21.76%, of the product is in good agreement with the findings of Keppler *et al*. (1959) while the purity shows some variation with the results reported by him. The urea complex of linoleic acid was analyzed by determination of iodine value of the complex (Knight *et al*., 1952). The results are reported in Table 3. The average iodine value of 160.1 shows a small variation from that reported by Keppler *et al*. (1959). It was been observed that the tendency of fatty acids and esters to combine with urea decreases with increasing unstauration.

Table 3. Percentage yield of linoleate (quantity and purity)

Iodine value	Purity of (%)	Yield of linoleate (%)
160.8	93.5	22.8
159.9	93.0	21.5
159.6	92.8	21.0
160.1*	93.1*	21.76*

* = average of triplicate analysis

It is thus concluded that omega-6 fatty acids, can well be extracted from the seeds of *C. tinctorius* and are suitable for use in nutritional products after purification.

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Major Ion Chemistry of Groundwaters From the Peshawar Intermontane Basin, NWFP, Pakistan

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Abstract. Investigation of spring and well waters of the Peshawar Intermontane basin and its surroundings in the Himalayan belt of Pakistan revealed that their temperature, pH, total dissolved solids and electrical conductivity values fall within the permissible range of drinking and irrigation water except the waters in vicinity of exposed faults. Minerals in the spring water are in the order of Ca > Mg > (Na + K) with bicarbonate as the dominant anion, whereas in the well water, the order is reversed, with sulphate as the dominant anion.

Keywords: Peshawar hydrochemistry, ground water, minerals, Pakistan

Introduction

Investigation was carried out to determine the field characteristics and major ion chemistry of spring and well waters. The study site covered the Peshawar intermontane basin (PIB) and its surroundings in the Himalayan foreland fold-and-thrust belt of Pakistan (Fig. 1). The area, located between latitudes 33.5°N-34.7°N and longitudes 71°E-73°E, is characterized by steep topography and V-shaped fluvial valleys in the north, which are drained by the river Indus entering from Indian Kashmir and the river Kabul entering from Afghanistan. Cold winters and warm, dry summers characterize the climate of the study area. June through August are the hot months, during which the daily mean maximum air temperature is about 40 °C. The mean annual potential evaporation ranges from 85 cm in the northern part of the study area to 130 cm in the centre of the basin. Snowfall occurs in the mountainous north during the cold months of December to February, when monthly mean minimum temperatures are several degrees Celsius below the freezing point.

Peshawar valley is home to two million plus inhabitants with ever growing demand for water for drinking, industrial and irrigation purposes. Every water usage (human, industrial, irrigation etc.) requires a set of hydrochemical characteristics, suited for that particular consumption. The hydrogeo-logical data for the region is scattered and scanty (Shah and Tariq, 2007; Tariq, 2001) and this research is the first attempt to fill this gap. The broader study conducted for this research is the first of its kind in that it synthesizes field, laboratory and simulation data to bracket the hydrochemical speciation in the PIB. This paper presents the field and laboratory data for groundwater in the study area.

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General geology. Information on subsurface geology of the study area has been derived from the lithological logs of boreholes drilled by WAPDA, Pakistan, and made available through various information releases. These data indicate that the quaternary sediments vary in thickness from few meters to more than 150 m. However, the total thickness of quaternary sediments is not known because none of the boreholes penetrates the bedrock. The coarseness of these sediments increases from north to south in the basin (Tariq, 2001). In the central part of the basin, the alluvial sediments consist of a relatively large proportion of fine-grained material, where the sandy silt is interbedded with discontinuous alluvial sand and thin gravel layers of various thickness. The main rock types in this area are slates, phyllites, various types of schists, paragneisses, sandstones, and quartzitic crystalline conglomerates, all of which are intruded by basic-to-acidic igneous rocks.

Hydrogeology. The study area can be divided into two hydrogeological provinces. The basin is bordered by mountains of the Lesser Himalayas on three sides while the south-eastern side is a fluvial valley carved at the confluence of the Indus and Kabul rivers. In the mountainous region, the water table varies in depth in different intermontane valleys, suggesting hydraulic discontinuities. In fact, water table elevation varies considerably within the area, ranging from less than 100 m in the basin centre to more than 1600 m in the mountainous north in relation to mean sea level (Fig. 2). Abundant springs (both normal and high temperature) are present in this part of the study area, and locally they constitute an important source of drinking water in addition to dugwells. The outflow from these springs ranges from less than one l/sec to more than 2000 l/sec. The field investigations indicate that most of the springs have good quality water, and according to local authorities, there are no water-

borne diseases in these areas. Most of these springs flow from unconsolidated or semi-consolidated fluvial deposits or talus surrounded by various types of hard rocks. The PIB sediments consist of mainly sand and gravel formations that serve as productive aquifers in the north and south of the basin. The main sources of recharge to the aquifers are precipitation, seepage from rivers, surface storage reservoirs and irrigation networks. A large number of drilled wells and dug wells are present throughout the PIB. These wells are extensively used for irrigation, industrial and domestic purposes. Drilled wells range in depth from 50 to 150 m, whereas the wells dug are up to 20 m in maximum depth.

Khyber, Attock-Cherat and Lower Swat-Bunner piedmont aquifers occur on the periphery of the basin, whereas flood plain and lacustrine aquifers occupy the central part of the basin. Depth to the water table is less than 5 m, except on the margins of the basin and in the southeast where it ranges from 5 to more than 30 m. Hydraulic conductivity ranges from 30-60 m/day and the average specific yield is 12% (Rathur, 1987); these values indicate a potentially high-yielding aquifer with substantial storage capacity. There is general groundwater flow from the margins of the basin towards the centre, with an average gradient of 0.004 (Bundschuh and Balke, 1991). Robberts (1988) estimated the total recharge to

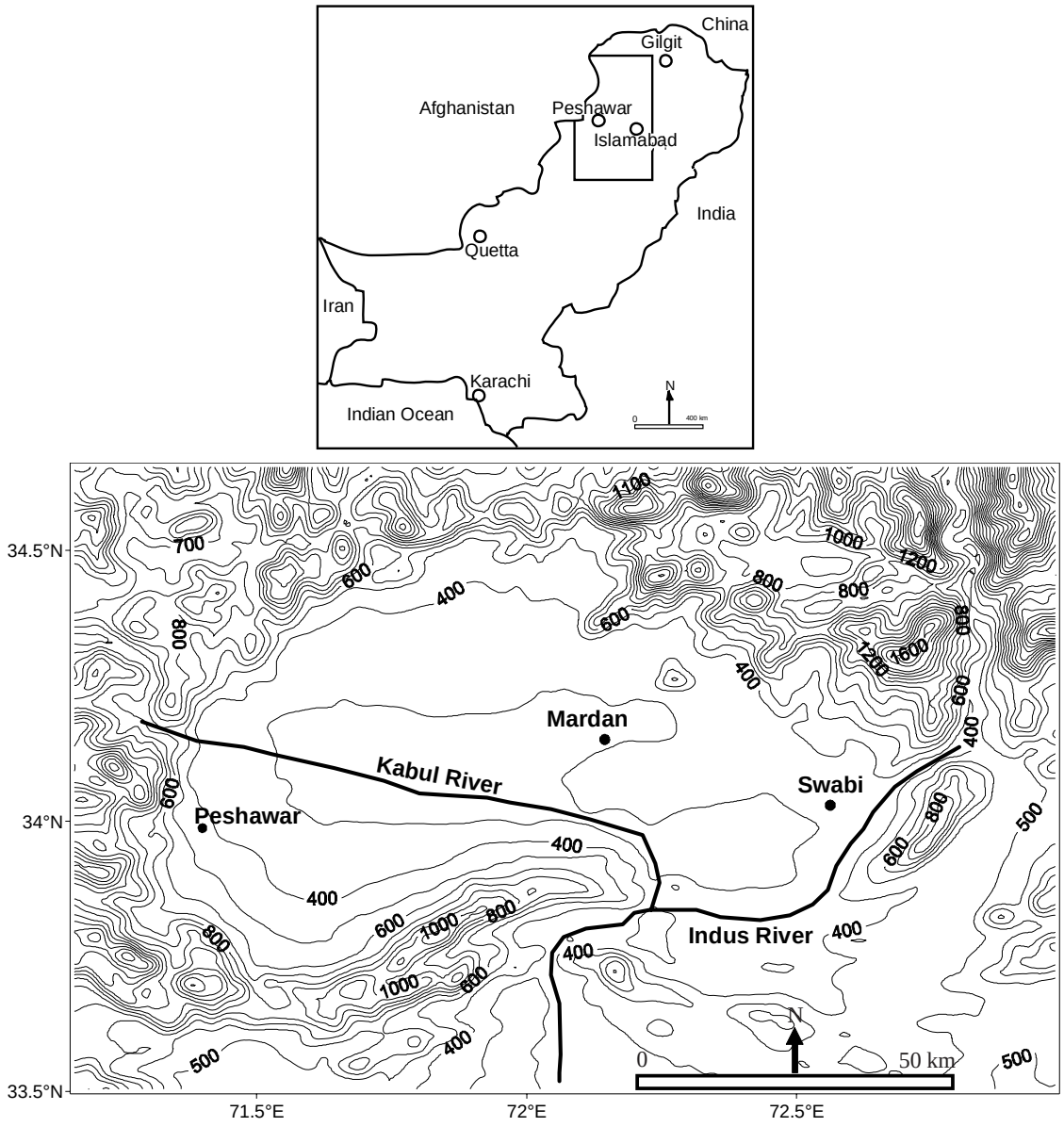

Fig. 1. Topographic map of the Peshawar Intermontane Basin (PIB) showing major localities and rivers. Contour elevations are shown in meters.

the basin as 923 Mm^3/year over a 6270 km^2 area. The main contributors to this recharge are precipitation (151 Mm^3/year), surface water irrigation (734 Mm^3/year), groundwater-based irrigation (15 Mm^3/year), and runoff water (23 Mm^3/year). The discharge of the groundwater takes place mainly along the downstream part of the Kabul river and its tributaries (Fig. 2). Additional discharge occurs through subsurface outflow into the basin, pumping for irrigation, drainage, and water consumption for domestic and industrial purposes. The total estimated discharge from the basin is 891 Mm^3/year, of which base flow constitutes 713 Mm^3/year (Rathur, 1987).

Materials and Methods

A total of two sets of 71 water samples were collected from water wells, springs and seepages throughout the study area and data from 32 samples spanning the PIB are presented in this paper (Fig. 2). Each sampling site was mapped with a Garmin GPS V with a position accuracy of less than ± 3 m. General physicochemical parameters were determined in the field using Technika Water Quality Meter 850081 with dedicated sensor probes. All the water samples were filtered in the laboratory with a 0.45 µm pore diameter filter, and the samples for cation analyses were acidified with nitric acid. Cations

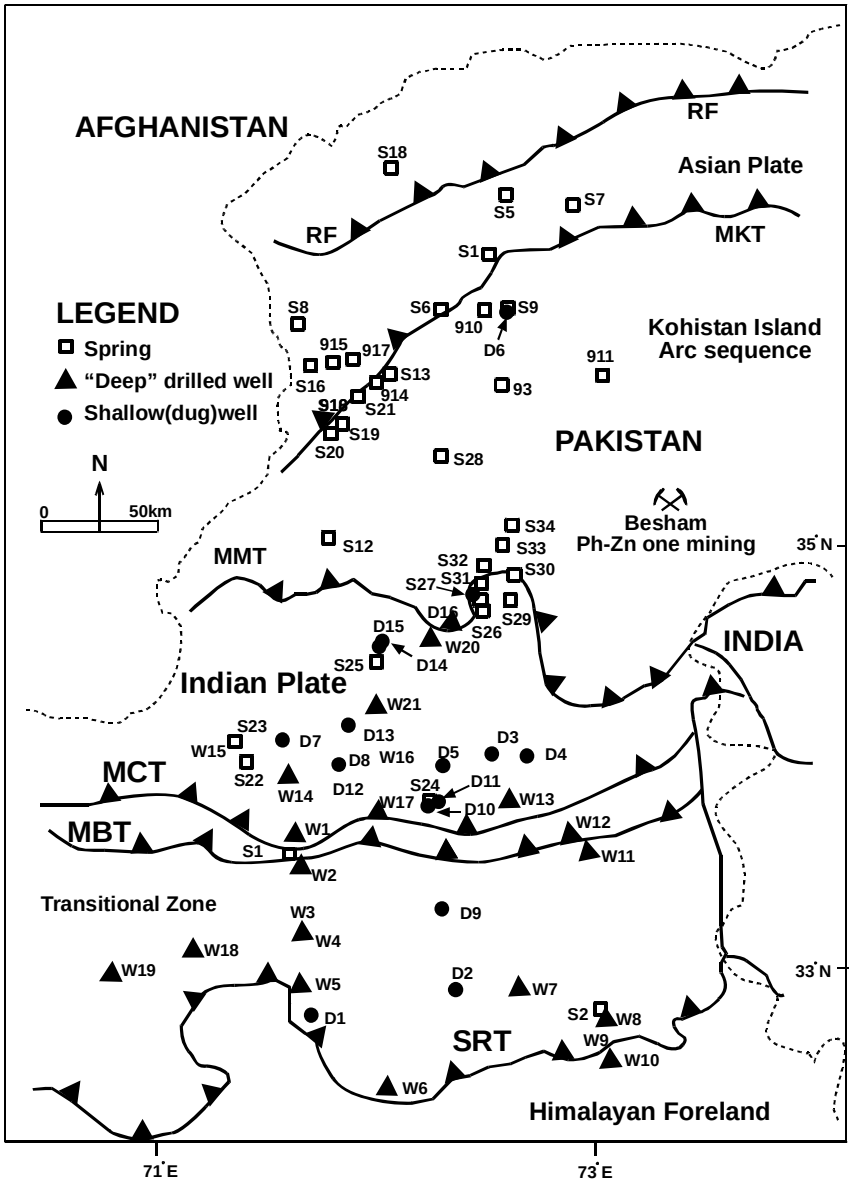

Fig. 2. Index map of sampling sites. Salt Range Thrust (SRT); Main Boundary Thrust (MBT); Kalabagh Fault (KF); Main Central Thrust (MCT); Main Mantle Thrust (MMT); Jhelum Fault (JF); Main Karakoram Thrust (MKT); Indus River (IR); Kabul River (KR); Kohistan Island Arc (KIA). PIB is located between MBT and MMT.

were analyzed using Perkin Elmer Optima 3300 DV ICP-OES with an AS90 plus autosampler. Precision errors were less than ± 6% for trace elements and less than ± 3% for the major cations (95% confidence). Multi-element stock solutions of 20 µg/ml were used as standards for trace elements. Standard solutions for major elements consisted of 1000 mg/l of single-element solutions. Anion concentrations were determined using dionex DX 120 ion chromatograph with an AS50 autosampler. Precision errors were less than ± 3% for all the anions (95% confidence). Anion standard solutions consisted of 1 mg/l, single-element solutions. Bicarbonate ion (HCO_3^-) was indirectly derived from total carbon analyzed by Shimadzu TOC 5000 instrument with precision error less than ± 2% (95% confidence). The charge balance (cations-anions/cations + anions) was less than ± 5% for all the samples.

Temperature, pH, total dissolved solids (TDS) and electrical conductivity (EC) were measured as part of the hydrochemical field work for this research.

Results and Discussion

The physicochemical data for springs and deep and shallow water-wells are presented in Table 1 and 2 alongwith the hydrochemical data. These values fall within the accepted range of values recommended for water used for drinking and irrigation purposes.

Water surface temperature. Temperature ranges from 14 to 29.5 °C for the well waters and from 8 to 38 °C for the spring waters. Groundwater temperature generally equilibrates itself with the mean annual air temperature for a particular locality. The mean annual air temperatures in the study area range from 13 to 25 °C (NASA, 2005). The regional distribution of differences between the water surface temperature and mean annual air temperature, designated here as ΔT °C, is given in Table 1 and 2. It can be noticed that most groundwaters show some surplus over the mean annual air temperature. This surplus is a result of the deep circulation of the regional groundwaters to a depth of about 4 km (Yousafzai *et al.*, 2006).

pH distribution. The values of pH exhibit a rather narrow distribution in the study area, as shown in Table 1 and 2. In particular, the well waters range in pH from 7.1 to 8.6, whereas pH values of the spring waters range from 6.7 to 8.7. Moreover, the groundwater tends to be more acidic away from most major fault zones (except the MCT and MBT) and more alkaline groundwater in the vicinity of these fault zones (Yousafzai *et al.*, 2006). Also, higher values of pH are noticed in the centre of Peshawar basin (> 7.8) as compared to the low values on its margins (< 7.6).

Conductivity and total dissolved solids. Conductivity, which is also known as electrical conductivity (EC), specific conductivity or conductance, is the reciprocal of the resistance in ohms between the opposite faces of a one cm cube of an aqueous solution at 25 °C. The International Unit for conductivity is the Siemens, which is numerically equivalent to the

Table 1. Hydrochemical data for groundwater samples from drilled and dug wells (concentration in mg/l)

Sample ID	Ca	Mg	Na	K	Cl	NO_3	SO_4	HCO_3	pH	TDS	EC (µS/cm)	ΔT °C	Balance
KPW-01	127.8	42.5	54.4	3.4	147.1	168.5	148.6	198.3	7.3	810	1210	6.3	-3.3
KPW-21	41.4	11.8	20.2	2.7	6.6	27.4	11.6	210	7.7	254	380	2.9	-4.0
PSW-51	57.6	25.1	34.6	3.8	50.6	39	87	230.1	7.6	423	632	6.9	-5.6
PSW-55	65.9	21.3	40.8	2.4	72.4	97.4	63.3	161.9	7.4	453	680	5.9	-3.9
PSW-57	38.1	16.2	56.3	4.3	14.2	16	32.3	285.4	7.5	353	530	6.6	-1.8
PSW-61	110.6	21.2	35.7	3.3	59.3	116.3	89.2	158.2	7.5	534	801	7.3	5.5
CSW-71	64.9	6.7	10.5	4.4	5.5	12.7	47.7	231.3	7.5	284	426	6	-4.3
PSW-22	75.6	22.3	23.7	4.2	27.9	57.4	129.3	203.6	7.6	441	431	5.1	-3.8
PSW-23	29	11.9	26.4	4.8	8.5	14.3	13.8	183	7.9	218	328	5.7	-0.7
PSW-24	18.3	10.3	27.6	5.7	11.2	10.2	15.2	152.3	7.7	198	297	4.7	-2.9
PSW-53	61.2	63.5	115.6	14.8	29.9	7.7	392.7	400.6	7.6	709	1061	1.3	-3.8
PSW-60	54	23.7	20.6	2.5	15.1	17.9	87.4	202.9	7.4	335	504	6.6	-2.1
PSW-62	23.6	3.4	6.3	3.2	5.5	4.3	40.4	60.1	8.1	101	151	8.1	-5.8
PSW-67	14.8	21.6	55	5.4	9.6	9.2	29.8	283.4	8	308	463	5.7	-5.9
CSW-69	70.6	7.3	7.3	2.4	12.6	6.2	10.4	205.3	7.7	345	522	6.4	5.6
CSW-70	62.9	8.9	3.3	2.1	4.6	5	38.3	228.4	7.5	272	408	6.8	-4.2
CSW-72	139.3	26.7	35.5	2.5	101.8	104.4	110.7	238.7	7.3	765	1148	6.6	0.0

Table 2. Hydrochemical data for groundwater samples from springs (concentration in mg/l)

Sample ID	Ca	Mg	Na	K	Cl	NO_3	SO_4	HCO_3	pH	TDS	EC (µS/cm)	ΔT °C	Balance
CSS-38	68	25.7	7.8	2.5	2.8	8.7	129.8	164.3	7.2	417	623	0.2	3.2
CSS-48	29.2	7.1	9	3.5	0.4	5.4	23.7	127.9	8.2	121	182	0.5	-4.2
PSS-54	59.3	23.5	15.8	1.8	17.3	36.2	74.1	219.4	7.3	351	527	7.7	-4.8
PSS-56	266.7	55	58.4	11.5	12.4	4.8	962	145.8	7.6	982	1475	5.7	-5.3
PSS-59	52	18.6	26.4	2.3	6.8	12.5	114.9	184.7	7.8	311	467	1.9	-4.2
CSS-68	95.4	29.1	14.8	3.4	8.5	7.8	252.1	178.3	7.6	372	559	2	-4.1
CSS-73	56.8	15.6	6.6	1.3	3.7	4.2	29.1	195.9	8.1	255	382	0.5	4.3
CSS-74	82.5	15.8	13.7	4.7	18.3	52.6	56.1	208.3	7.3	389	584	1.2	1.6
CSS-75	55.6	12.4	8.6	2.7	10.1	14.4	27.9	211.8	7.4	266	402	0.7	5.0
CSS-76	72.6	3.1	4.5	0.5	6.2	11.2	5.4	166.6	7.3	303	457	3.7	4.7
CSS-78	6.1	0.3	4.1	1	1.4	7.4	4.2	3.4	7.5	27	41	2.8	5.2
CSS-79	42.2	24.5	6.9	1.1	3.1	16.7	9.5	249.5	7.8	264	396	2.6	-2.0
CSS-80	27.8	6.1	6.2	1.4	6.6	4.8	13	84.1	6.7	124	182	1.1	5.7
CSS-81	30.4	9.8	7.8	2.2	6.3	17.3	12.5	113.3	7.2	152	227	1	2.8
CSS-82	10.2	0.9	2.9	1.4	3.3	4	4.6	26.3	8.1	27	40	0.6	4.7

mhos (Hounslow, 1995). The EC values indicate a general impression of the chemical behaviour and chemical quality of the groundwater in an area and are linked to the quantity of salts dissolved in the groundwater (Bundschuh, 1992). A usual rule for drinkable water is 10 mhos or 1000 micro Siemens per cm (µS/cm). The EC values range from 151 to 1210 µS/cm in the well waters, and from 40 to 584 µS/cm in the spring waters with the exception of one sample (S2, Fig. 2) which shows an EC value of 1475 µS/cm.

The highest values of EC are clustered around two centres in the Kohat-Pothohar basin. Groundwater in the vicinity of these centres is salty in taste and may cause longterm health problems. Generally, the distribution of EC values corresponds to the depth of groundwater levels; that is, the shallower the groundwater, the higher the conductivity (Bundschuh and Balke, 1991). No such correlation was observed in the groundwater of the studied area, however. The use of highly mineralized groundwater for irrigation purposes in some parts of the study area may lead to an increasing salt content of both the soil and groundwater (Bundschuh and Balke, 1991). Total Dissolved Solids (TDS) is calculated by adding the mass of ions to that of SiO_2. TDS ranges from 101 to 810 mg/l in the well waters and from 27 to 982 mg/l in the spring waters (Table 1-2).

Regional abundance of major elements in the groundwater samples are presented in Table 1-2, and the analyses are plotted on discriminant diagrams in Fig. 3 and 4. Conclusions can be drawn from these discriminant diagrams regarding water type, precipitation or solution, mixing and ion exchange (Hounslow, 1995). The diamond part of the diagram may be used to characterize different water types. Most of the spring water is typically characterized by elemental abundance in the sequence Ca>Mg>(Na+K), with bicarbonate as the dominant anion, suggesting young and fresh recharge. However, two samples exhibit (Na+K)>Ca>Mg, with sulphate as the dominant anion (Fig. 3); both samples also show anomalous temperatures. The linear trend on the discriminate diagram for spring water- taken together with high TDS values indicate

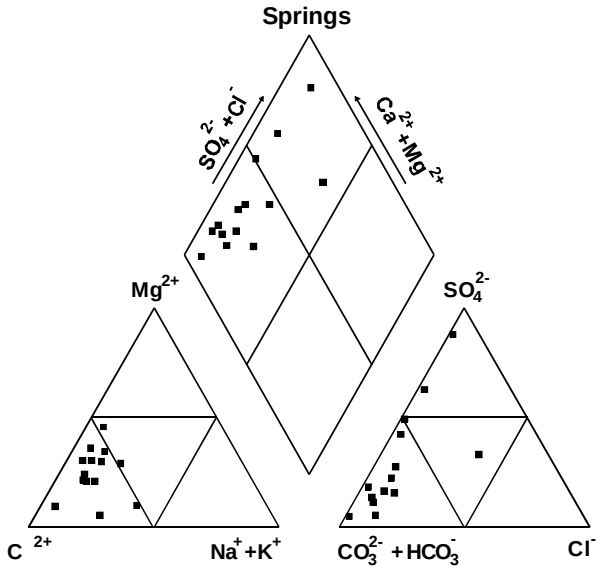

Fig. 3. Discriminate diagram for spring water samples.

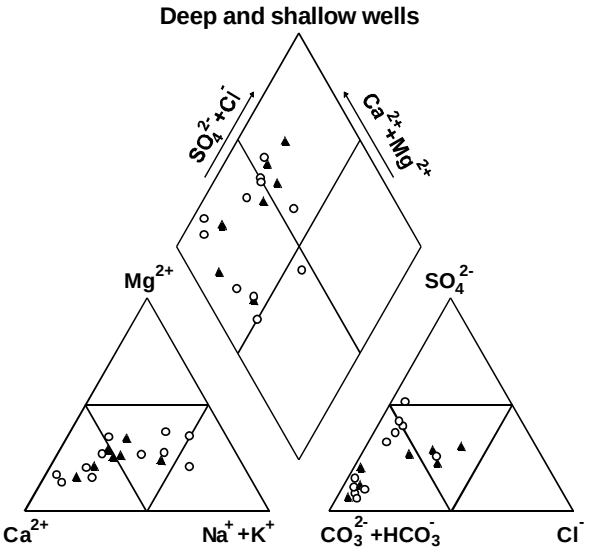

Fig. 4. Discriminate diagram for well water samples. Open circles represent shallow wells while deep wells are shown by triangles.

dissolution of Ca^{2+}, Mg and sulphate (Fig. 3). Examination of Table 2 indicates that Ca^{2+} is the dominant cation in these samples, suggesting dissolution of chlorites and related rocks which are abundant in the region. The linear trend stretches all along the left axis of the plot, indicating mixing of two types of waters.

The trends for both the shallow and deep water samples exhibit ion exchange in which calcium and magnesium in solution are being replaced by sodium. The trends start parallel to constant magnesium and then curve towards the sodium apex, suggesting that more calcium is being exchanged than magnesium (Hounslow, 1995). Most significantly, water samples from one shallow well and three deep wells, all located in an immediate vicinity of the major thrust zones (MBT and MMT), demonstrate clear imprints of admixture of oil-brines. All of these samples also show a significant surplus temperature over the local mean annual ground surface temperature (Table 1-2). The surplus temperature over the local mean annual ground surface temperature (ΔT °C) shows several positive anomalies. One such anomaly is located along the MKT, and another is located along MCT and MBT. All of these samples exhibit temperatures of at least 6 °C above the local mean annual temperature (Table 1-2). Furthermore, all samples with such significant excess temperature over the local mean annual air temperature also have anomalously high concentrations of SiO_2, whereas those along the MKT are also characterized by anomalously high concentrations of boron and strontium (Yousafzai *el al.*, 2008).

Conclusion

Most of the Peshawar Basin aquifers are unconfined. Temperature, pH, total dissolved solids (TDS), and electrical conductivity (EC) values of the groundwaters fall within the normal range for water used for drinking and irrigation purposes with the exception of a few samples (KPW-01, PSW-53, CSW-72, PSS-56). These samples represent waters which are in the vicinity of exposed or blind faults and may indicate thermal waters which have excessively dissolved materials while ascending to shallow levels. Results from water chemistry indicate that the spring water is dominated by the sequence Ca > Mg > (Na+K), with bicarbonate as the dominant anion, suggesting young and fresh recharge. However, the well water exhibits the reverse order of preference for these cations indicating the process of ion exchange. Cation exchange processes play an important role in controlling the chemical composition of groundwater in addition to dissolution processes (Grasby *et al.*, 1999; Cerling *et al.*, 1989). There are several possible explanations for the variation in the groundwater chemistry such as the mixing of waters of different origins, dissolution of the clastic sediments composing the aquifer systems, precipitation of minerals from the water and cation exchange with clay etc.

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Short Communication

Determination of Positional Isomers of Monoenoic Fatty Acids Separated From Seed Oils of *Nicotiana tabacum* L. and *Nicotiana rustica*

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Abstract. The octadecenoic acid separated from the seed oils of *Nicotiana tabacum* L. and *Nicotiana rustica* by the application of argentation thin layer chromatography was oxidized by modified Von Rudloff's technique. The liberated monofunctional and difunctional carboxylic acids were separated and identified by the application of thin layer chromatography and gas chromatography. The positional isomers determined in both species were *cis*-9-octadecenoic acid and *cis*-11-octadecenoic acid.

Keywords: isomers, monoenoic fatty acids, *Nicotiana tabacum, Nicotiana rustica,* seed oils

Nicotiana tabacum L. and *Nicotiana rustica*, yield tobacco and are commonly cultivated as crops (Kirtikar and Basu, 1984). The monoenoic fatty acid i.e. octadecenoic acid separated from the oils of both the species is oxidized to determine the positional isomers. The literature reveals that octadecenoic acid of *N. tabacum* consists of two positional isomers (Frega *et al*., 1991) *cis*-9-octadecenoic acid and *cis*-11- octadecenoic acid, whereas no work has been reported on the positional isomers of *N. rustica*. The present work reports the positional isomers of *N. tabacum* and *N. rustica.*

Liberation, methylation and purification of fatty acids from the plants were carried out according to the methods described by Waheed *et al.* (2001), Raie *et al.* (1989) and Devine and Williams (1961). The purified methyl esters were separated into four fractions (I to IV) with $AgNO_3$ impregnatede thin layer chromatography.

Separation and oxidation of octadecenoic acid were carried out by the methods described by Chaudri *et al*. (2001), Hamilton and Raie (1972) and Von Rudloff (1956).

The mixture of mono- and di-carboxylic acids produced in aqueous media was analyzed by gas chromatography using porapak "Q" column and short chain mono-carboxylic acids (C_4-C_9) were identified and later, the remaining acids (above C_{10}) were analyzed as described by Raie *et al.* (1989). The methyl esters of mono- and di-carboxylic acid were separated by preparative silica gel plates using mixture of hexane: diethyl ether (1 : 1 v/v) as a developing solvent.

The purified methyl esters of mono-carboxylic acid (C_9 and C_{11}) were analyzed and identified by the use of 5% SE-30 packed column at 110 °C to 150 °C and those of di-carboxylic acids were analyzed at 150 °C to 200 °C. Their identification was confirmed with the help of standards. The R_f values of the separated four fractions of fatty acids of the two species are reported in Table 1 and their composition in Table 2.

The percentages of saturated fatty acids and monounsaturated fatty acids in *N. tabacum* and *N. rustica* were 12.7% and 12.3%, respectively, whereas, the percentages of diunsaturated and triunsaturated fatty acids were 73.9% and 71.6% and 1.2% and 0.9%, respectively, in both the species (Table 2).

Table 3 and 4 show that the two positional isomers of the monoenoic fatty acid (octadecenoic acid) are present in the seed oil of *N. tabacum* i.e., *cis*-9-octadecenoic acid (11.1%) and *cis*-11-ctadecenoic acid (1.1%); similarly two positional isomers of monoenoic fatty acid (octadecenoic acid) i.e. *cis*-9-octadecenoic acid (14.4%) and *cis*-11-octadecenoic acid (0.8%) are present in *N. rustica.*

The oxidation of monounsaturated fatty acid (octadecenoic acid) was carried out to cleave double bonds and eventually to produce monobasic and dibasic acids, which helped to determine the positional isomers. Since complications arise by the production of a number of monobasic and dibasic

Table 1. R_f values of different fatty acid bands (as methyl esters) separated by the $AgNO_3$-TLC

Band no.	Bands	Fatty acid	R_f
I	saturated acids	$C_{12:0}$, $C_{14:0}$, $C_{16:0}$ $C_{18:0}$, $C_{20:0}$	0.65
II	monounsaturated acid	$C_{18:1}$	0.51
III	diunsaturated acids	$C_{18:2}$	0.30
IV	triunsaturated acids	$C_{18:3}$	0.18

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acids after the oxidation of polyunsaturated fatty acids, therefore, the oxidation technique was applied exclusively for monoenoic fatty acid although the diunsaturated fatty acids are present in large amounts as compared to monosaturated fatty acid in both the species of *Nicotiana* (Table 2).

A number of oxidizing agents (Bailey, 1958; Rigby, 1956; Swern, 1948; Milas, 1937) have been used for the cleavage of double bonds but potassium permanganate and sodium metaperiodate had been claimed to be the best oxidizing agents and the method was known as modified Von Rudloff's oxidation (Hamilton and Raie, 1972). The oxidation of unsaturated fatty acids by permanganate did not normally involve any change of configuration and the procedure had been applied to many other seed oils (Galun *et al.,* 1984; Hammond *et al.,* 1972; Wilson *et al.,* 1962).

Table 2. Percentage of different fatty acids (as methyl esters) separated on the $AgNO_3$-TLC

Band no.	Fatty acids	*N. tabacum* (%)	*N. rustica* (%)
I	saturated	12.7	12.3
II	monounsaturated	12.2	15.2
III	diunsaturated	73.9	71.6
IV	triunsaturated	1.2	0.9

Table 3. Positional isomers of octadecenoic acid of the oil of *N. tabacum*

Chain length of monobasic acid	Chain length of dibasic acid	Position of double bond	Percentage	Isomers	Referred work* percentage
C_9	C_9	Δ_9	11.1	*cis*-9-octa-decenoic	10.6
C_7	C_{11}	Δ_{11}	1.1	*cis*-11-octa-decenoic	0.6

* Frega *et al*., 1991

Table 4. Positional isomers in octadecenoic acid of the oil of *N. rustica*

Chain length of monobasic acid	Chain length of dibasic acid	Position of double bond	Percentage	Isomers
C_9	C_9	Δ_9	14.4	*cis*-9-octa-decenoic
C_7	C_{11}	Δ_{11}	0.8	*cis*-11-octa-decenoic

Since the major problem was the analysis of the water-soluble short chain acids produced as a result of the oxidation of monoenoic fatty acid, therefore, short chain acids in aqueous media were analysed directly on a column of Pora-Pak Q. The short chain mono-carboxylic acids and di-carboxylic acids produced by the oxidation of monoenoic fatty acid separated from the oil of *N. tabacum*, showed that there are two positional isomers i.e. *cis*-9-octadecenoic acid and *cis*-11-ctadecenoic acid showing double bond at $\Delta_{9:10}$ and $\Delta_{11:12}$, respectively (Table 3). The literature (Frega *et al.,* 1991) revealed that there were only two positional isomers among the monounsaturated fatty acids i.e. *cis*-9- octadecenoic acid commonly known as oleic acid (11.1%) and *cis*-11-octadecenoic acid usually known as vaceenic acid (0.7%). Similarly the positional isomers of *N. rustica* are *cis*-9-octadecenoic acid (14.4%) and *cis*-11-ctadecenoic acid (0.8%) having the double bond at $\Delta_{9:10}$ and $\Delta_{11:12}$ (Table 4). These studies reflect that positional isomers have a definite biosynthetic relationship.

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Stimulatory Effect of Medium Ingredients on Alkaline Protease Production by *Bacillus licheniformis* N-2 and Compatibility Studies With Commercial Detergents

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Abstract. Suitable concentration of ingredients of the growth medium played a vital role in production of alkaline protease by *Bacillus licheniformis*. Maximum enzyme activity (875.05 PU/ml) was achieved when the bacterium was grown in the medium containing glucose (1%), soybean meal (1%), K_2HPO_4 (0.5%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.05%), $CaCl_2.2H_2O$ (0.05%) at 37 °C on 24 h incubation period with agitation of 140 rpm in shake flask cultures. More than 1% glucose decreased the enzyme production. The protease had excellent stability with wide range of commercial detergents such as Ariel, Bonus, Bright Total, Surf Excel, Wheel and non-branded detergents, recommending its use as an effective additive in detergent formulation.

Keywords: medium ingredients, detergent compatibility, *B. licheniformis* N-2, alkaline protease

Introduction

Proteases are one of the most important groups of industrial enzymes used in pharmaceutical industry and in food industry for peptide synthesis, in leather industry for de-hairing and in detergent industry as an additive of detergent formulation (Joo and Chang, 2005; Pastor *et al.,* 2001). Alkaline proteases are known to constitute 60-65% of the global industrial market among various types of proteases (Banerjee *et al.,* 1999). Alkaline proteases are produced by a wide range of microorganisms including bacteria, mould and yeast. Currently, a large portion of commercially available proteases is derived from *Bacillus* strains because of their high pH and temperature stability (Gupta *et al.,* 2002; Joo *et al.,* 2002).

The fermentation medium form the environment in which the microorganisms live, reproduce and carry out their specific metabolic reactions to produce useful products. Two distinct biological requirements are considered in most of the industrial fermentation processes for medium design, where the product is something other than the cell mass itself. First, the nutrient has to be supplied to establish the growth of the microorganism. Second, proper nutritional conditions have to be provided to maximize the product formation. It is also well established that extracellular protease production in microorganisms is greatly influenced by media components, especially carbon and nitrogen sources, metal ions and physical factors such as pH, temperature, dissolved oxygen and incubation time (Nadeem, *et al*. 2006; Oberoi *et al.,* 2001; Kuar *et al.,* 2001; Razak, *et al.* 1994; Moon and Parulekar, 1993). The cost of the growth medium is another significant parameter for making the production process industrially viable. Approximately 30-40% of the production cost of the industrial enzyme is estimated to be accounted for by the cost of the growth medium (Gessesse, 1997). Therefore, selection of the right medium ingredients and their concentrations optimization have become the need of today for high yield of desirable enzymes by fermentation.

Considering these facts, the effects of different concentrations of carbon and nitrogen sources as well as metal ions concentrations were studied to maximize the yield of alkaline protease by locally isolated *Bacillus licheniformis* N-2. Compatibility studies of alkaline protease with different detergents were also conducted to observe its commercial exploitation as an additive in detergent formulation for laundry industry.

Materials and Methods

Microorganism and culture maintenance. A proteolytic strain identified as *Bacillus licheniformis* N-2 was isolated from decaying organic soil sample (Nadeem *et al*., 2007) and the culture was grown on nutrient agar slants at 37 °C for 24 h and preserved at 4 °C for one month. The preserved culture was revived on fresh nutrient agar slants after every one month for subsequent experiments.

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***Inoculum preparation*.** The inoculum was prepared by transferring a loopful of 24 h old culture of *B. licheniformis* N-2 into 50 ml of inoculum medium consisting of glucose (0.5%), soybean meal (1%), K_2HPO_4 (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.05%), and $CaCl_2.2H_2O$ (0.05%). The inoculated medium was incubated in water bath shaker (Eyela, Japan) for 24 h at 37 °C temperature and 140 rpm agitation speed for the propagation of bacteria up to 10^{8-10} cells/ml.

Fermentation process. The initial growth medium composed of glucose (1%), soybean meal (1%), K_2HPO_4 (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.05%) and $CaCl_2.2H_2O$ (0.05%). It inoculated with 1% (v/v) of 24 h old inoculum broth. The pH of the medium was adjusted at 10 with 1N HCl/NaOH before sterilization at 121°C for 15 min. The inoculated medium was incubated in water bath shaker (Eyela, Japan) for 24 h at 37 °C and 140 rpm. Thereafter, the fermented broth was centrifuged at 9000 x g for 10 min at 4 °C to get clear supernatant enzyme solution.

Optimization of concentrations of medium ingredients. Different concentration levels of carbon and nitrogen sources as well as inorganic elements were optimized to make the process cost effective for alkaline protease production by *B. licheniformis* N-2.

***Effect of glucose concentrations*.** Effect of various glucose concentrations (0.2, 0.4, 0.6, 0.8, 1.0, 1.25 and 1.50%) were studied to find the suitable glucose concentration for the maximum yield of alkaline protease by *B. licheniformis* N-2.

***Effect of defatted soybean meal concentrations*.** Effect of different concentrations of defatted soy bean meal viz. 0.25, 0.50, 0.75, 1.0, 1.25, 1.5, 1.75 and 2.0% on alkaline protease production was determined by incubating the growth medium at 37 °C for 24 h in water bath shaker (Eyela, Japan) with agitation speed of 140 rpm.

***Effect of metal ions on alkaline protease production*.** Effects of various metal ions in the form of K_2HPO_4, $MgSO_4.7H_2O$, NaCl, and $CaCl_2.2H_2O$ on alkaline protease production were studied to find the suitable concentration level of each element in the growth medium. The concentration of each element was used in the range of 0.01% to 0.07% (w/v) except K_2HPO_4, with concentration varying from 0.1% to 0.7%. All the experiments were performed in triplicate by changing concentration of one element but keeping the concentration of others constant.

Analytical procedures: *Total protein contents*. Total protein contents of samples were determined by the method of Lowry *et al*. (1951) using bovine serum albumin (BSA) as the reference standard.

***Total biomass determination*.** The cell biomass was determined from a known amount of sample centrifuged at 9000 x g for 10 min at 4 °C. The cell biomass pellet was washed with sterilized normal saline three times to remove the suspended particles. The washed cell pellet was dried at 105 °C till the constant weight is obtained.

***Determination of alkaline protease activity*.** Proteases activity was determined by a slightly modified method of Yang and Huang (1994). The reaction mixture containing 1 ml of 1.0% casein solution in 0.05 M glycine-NaOH buffer having pH 11 and 1 ml of enzyme solution were incubated at 60 °C for15 minutes and the reaction was then stopped with 3 ml of 10% tri-chloroacetic acid (TCA). After 10 min the entire mixture was centrifuged at 9000 x g for 10 min at 4.0 °C and absorbance of the liberated tyrosine was measured with respect to sample blank at 280 nm. One proteolytic unit (PU) was defined as the amount of the enzyme that released 1µg of tyrosine under the assay conditions.

Compatibility of alkaline protease with various commercial detergents. The compatibility of *B. licheniformis* N-2 protease with different detergents was studied using Ariel, Bonus, Bright Total, Surf Excel, Wheel and non-branded detergent. All the detergents were diluted with distilled water to give final concentration of 7 mg/ml to stimulate washing conditions. The enzyme in the detergents were deactivated by heating the detergents solution at 100 °C for 10 min. After that, a known quantity of alkaline protease in the presence of 5 mM and 10 mM $CaCl_2$ was added to each detergent solution separately. The enzyme mixtures were incubated at 40 °C for different time intervals (15-120 min) and then the residual activity was determined according to the standard assay procedure. The enzyme activity was taken as 100% of the enzyme mixture without incubation.

Chemical and statistical analysis. All the chemicals used in this study were of analytical grade. Each experiment was conducted in triplicate and the standard deviation (SD) was calculated by using Microsoft Excel Program.

Results and Discussion

Effect of carbon concentrations on alkaline protease production. As glucose was found to be the best carbon source (un-published data) for alkaline protease production by *B. licheniformis* N-2, therefore, different concentrations of glucose varying from 0.2% to 1.5% over the control (without external source of glucose) were used to determine the optimum concentration for the maximum yield of alkaline protease. The highest protease production (678.21 PU/ml) and cell biomass (3.71 g/l) was observed at 1.0% concentration

(w/v) of glucose (Table 1). A decrease in enzyme production was observed at lower or higher concentrations other than the optimum. The results indicated that proper concentration level of glucose plays significant role in enhancing the production of alkaline protease and growth of the *B. licheniformis* N-2. Higher concentrations repressed the growth and enzyme production might be due to the catabolic repression, or substrate inhibition, a traditional property of the batch fermentation processs. A decrease in enzyme production was also observed at higher concentration of glucose (> 8 k/g m^3) by Calik *et al*. (2003). Adinarayana *et al.* (2003) found maximum enzyme yield at 0.5% glucose concentration by *Bacillus subtilis*. On the other hand, He *et al.* (2003) reported highest protease activity (2508U/ml) by *Bacillus* sp. EL31410 at 4 % glucose concentration level. The difference in glucose concentration among different *Bacillus* species might be due to the difference in physiological and metabolic characteristics. It is well known that there is big difference among the metabolic characteristics of the same species isolated from different sources.

Effect of soybean meal concentration on alkaline protease production. Among different nitrogen sources tested for alkaline protease production by *B. licheniformis* N-2 (data not shown), soybean meal exhibited a prominent effect on protease production and growth of the *bacterium*. Soybean meal had stimulatory effect on alkaline protease production and maximum yield (680.02 PU/ml) was obtained at 1.0% (w/v) concentration (Table 2). Further increase in concentration considerably repressed the growth and protease production. A similar finding was observed by Elibol and Moreira (2005) who observed maximum enzyme production at 1.0% concentration level by a marine bacterium *Teredinobacter turnirae*. Optimum alkaline protease yield was reported at 1.5% concentration of soybean meal in the growth medium

Table 1. Effect of glucose on alkaline protease production by *B. licheniformis* N-2 after 24 h incubation at 37 °C and 140 rpm

Glucose conc. (%)	Cell biomass (g/l)	Total protein (mg/ml)	Final pH of the medium	Enzyme activity (PU/ml)
Control	2.68±0.03	2.25±0.02	8.61±0.12	315.22±7.22
0.20	2.79±0.02	2.27±0.06	8.56±0.11	375.35±6.38
0.40	3.15±0.04	2.43±0.04	8.51±0.13	405.81±9.11
0.60	3.41±0.07	2.85±0.11	8.43±0.08	500.06±5.78
0.80	3.58±0.05	3.09±0.05	8.25±0.10	586.19±6.04
1.00	3.71±0.06	3.34±0.12	8.18±0.12	678.21±7.12
1.25	3.62±0.07	3.12±0.10	8.17±0.11	635.36±7.89
1.50	3.31±0.08	2.74±0.07	8.21±0.09	445.74±8.06

control: without external source of glucose; each value is an average of three parallel replicates ±indicates standard deviation among the replicates

Table 2. Effect of defatted soybean meal on alkaline protease production by *B. licheniformis* N-2

Soybean meal conc. (%)	Cell biomass (g/l)	Total protein (mg/ml)	Final pH of the medium	Enzyme activity (PU/ml)
Control	2.82±0.13	2.02±0.05	8.13±0.07	255.35±5.54
0.25	2.95±0.09	2.45±0.03	8.16±0.10	315.33±8.14
0.50	3.17±0.10	2.88±0.08	8.19±0.07	425.08±4.03
0.75	3.35±0.07	3.14±0.11	8.18±0.12	534.32±5.42
1.00	3.74±0.11	3.35±0.10	8.19±0.11	680.02±9.07
1.25	3.73±0.08	3.45±0.06	8.21±0.06	666.11±4.44
1.50	3.73±0.10	3.52±0.05	8.20±0.08	635.08±5.06
1.75	3.65±0.07	3.47±0.04	8.19±0.05	612.46±9.11
2.00	3.52±0.12	3.44±0.07	8.21±0.10	561.43±10.35

control: without external source of defatted soybean meal; each value is an average of three parallel replicates ± indicates standard deviation among the replicates

from *Bacillus* sp. I-312 and *Bacillus horikoshii* (Joo and Chang, 2005; Joo *et al.*, 2002). In their studies on protease production, Laxman *et al*. (2005) observed maximum yield at 2% soybean meal concentration from *Conidiobolus coronatus* while Sutar *et al.* (1992) reported highest protease activity in the medium containing 4% soybean meal. All these findings indicated that different concentration levels of soybean meal influenced the metabolic processes of each microorganism involved in fermentation process. Therefore, capacity of utilization of soybean meal varies from species to species or even in the same species isolated from different environments.

Effect of different metal ions on alkaline protease production: *Effect of K_2HPO_4 concentrations on alkaline protease production*. Effects of various metal ions on cell growth and enzyme production were studied stepwise for the optimum yield of alkaline protease by *B. licheniformis* N-2. For this purpose, the basal medium was amended with different concentrations of K_2HPO_4 varying from 0.1% to 0.7% against control (without external source of K_2HPO_4). Maximum alkaline protease yield (873.25 PU/ml) was observed at 0.5% initial concentration (w/v) of K_2HPO_4 that gave 2.1 fold increases in enzyme production over the control (Table 3). Theses results are in agreement with the earlier findings which showed enhancement of protease activity in the presence of metal ions (Adinarayana *et al.*, 2003; Thangam and Rajkumar, 2002). Our findings are also supported by Calik *et al.* (2003) who found 1.55 fold higher enzyme activity in the presence of phosphate ions by *Bacillus subtilis.* Rahman *et al*. (2005) observed 39% increases in enzyme production in the presence of 2mM K^{1+} ions in the growth medium by *Pseudomonas aeruginosa*. All these findings indicated that potassium and phosphate ions provided by K_2HPO_4 play an important role in growth of microbes and enzyme production because these metal ions are the major constituent of nucleotide, nucleic acid and phospholipids.

Effect of $MgSO_4.7H_2O$ concentration on alkaline protease production. Magnesium is an essential cofactor for many of the glycolytic enzymes and depletion of magnesium in the culture broth inhibits glycolysis (Dombek and Ingram, 1986). Therefore, various concentrations of $MgSO_4.7H_2O$ ranging from 0.01% to 0.07% were studied to observe the suitable concentration of Mg^{2+} ions for maximum growth and alkaline protease production by *B. licheniformis* N-2. The results revealed 1.1 fold increase in enzyme production (873.22 PU/ml) in the presence of 0.05% $MgSO_4.7H_2O$ concentration in the growth medium (Table 4). The results are supported by Jasvir *et al.* (1999) who observed increase in enzyme production in the presence of Mg^{2+} ions. Rahman *et al.* (2005) also observed 14% increase in enzyme production in the presence of 2mM Mg^{2+} ions. However, a decrease in alkaline protease production was observed by Calik *et al*. (2003) in the presence Mg^{2+} ion that might be attributed to the difference in medium composition as well as in the microorganism.

Effect of NaCl concentration on alkaline protease production. The effect of Na^{+} ions on alkaline protease production and growth of *B. licheniformis* N-2 was studied by amending the cultivation medium with different concentrations (w/v) of NaCl ranging from 0.01% to 0.07%. Maximum enzyme activity (874.61 PU/ml) was obtained at 0.05% concentrations of NaCl which indicated 1.16 fold increase in the enzyme yield as compared to the control (Table 5). Thereafter, a decrease in alkaline protease yield was observed with further increase in concentration of NaCl. Shanmughapriya *et al.* (2007) obtained highest protease activity at 3 % NaCl

Table 3. Effect of K_2HPO_4 on alkaline protease production by *B. licheniformis* N-2

K_2HPO_4 conc. (%)	Cell biomass (g/l)	Total protein (mg/ml)	Final pH of the medium	Enzyme activity (PU/ml)
Control	3.11±0.04	2.93±0.08	7.15±0.11	415.19±8.85
0.10	3.15±0.12	2.96±0.04	7.48±0.08	470.21±9.25
0.20	3.37±0.07	3.25±0.06	8.06±0.10	551.83±6.66
0.30	3.72±0.15	3.36±0.07	8.21±0.06	678.93±8.12
0.40	3.81±0.10	3.42±0.03	8.33±0.09	725.43±11.05
0.50	3.84±0.08	3.45±0.06	8.54±0.04	873.24±12.67
0.60	3.82±0.06	3.47±0.05	8.67±0.06	678.36±8.98
0.70	3.78±0.12	3.46±0.05	8.81±0.08	619.54±10.08

control: without external source of K_2HPO_4; each value is an average of three parallel replicates ± indicates standard deviation among the replicates

concentration by a marine isolated *Roseobacter* sp. This big variation with our findings might be due to difference in the nature of the species.

Effect of $CaCl_2.2H_2O$ concentration on alkaline protease production. Effect of Ca^{2+} ions on protease production and growth of *B. licheniformis* N-2 were investigated by using $CaCl_2.2H_2O$ as a Ca^{2+} ion source of varying concentration from 0.01 to 0.07% over control (without external source of $CaCl_2.2H_2O$). The results revealed that 0.05% concentration affected the growth and protease production noticeably and enhanced 1.3 fold enzyme yield over the control (Table 6). Ca^{2+} ions were studied as an effective inducer for enzyme production by Shafee *et al.* (2005). Vidyasagar *et al.* (2006) investigated the similar findings and observed maximum enzyme production at 200mM Ca^{2+} ions by *Halogeometricum* sp. TSS101. Our results are also in good agreement with Mabrouk *et al.* (1999) who observed 26.6% increase in enzyme production in the presence of 0.07% $CaCl_2.2H_2O$ over the control in the growth medium. All these finding indicated that Ca^{2+} ions had stimulatory effect on protease production and the presence of Ca^{2+} ion in the fermented broth might also stabilize the structure of extracellular alkaline protease.

Compatibility studies with various commercial detergents. The compatibility of alkaline protease with different commercial detergents (brand names: Arial, Bonus, Bright Total, Wheel and un-branded detergent) were studied in the absence and presence of different concentrations (5 mM and 10 mM) of $CaCl_2.2H_2O$. Alkaline protease from *B. licheniformis* N2 showed a wide range of compatibility with different detergents. Maximum compatibility was observed with Bright Total and Wheel up to 58% and 58.06% after 120 min of incuba-

Table 4. Effect of $MgSO_4.7H_2O$ on alkaline protease production by *B. licheniformis* N-2 after 24 h incubation at 37 °C and 140 rpm

$MgSO_4.7H_2O$ conc. (%)	Cell biomass (g/l)	Total protein (mg/ml)	Final pH of the medium	Enzyme activity (PU/ml)
Control	3.47±0.08	3.37±0.03	8.53±0.08	795.12±10.02
0.01	3.54±0.10	3.38±0.05	8.52±0.11	815.46±12.41
0.02	3.64±0.12	3.38±0.05	8.54±0.07	829.12±11.67
0.03	3.72±0.11	3.40±0.04	8.54±0.12	842.37±15.08
0.04	3.78±0.07	3.44±0.08	8.53±0.06	861.19±8.92
0.05	3.85±0.10	3.46±0.05	8.55±0.10	873.22±9.44
0.06	3.84±0.06	3.48±0.04	8.56±0.06	862.03±10.11
0.07	3.83±0.07	3.48±0.06	8.55±0.05	846.27±9.18

control: without external source of $MgSO_4.7H_2O$; each value is an average of three parallel replicates ± indicates standard deviation among the replicates

Table 5. Effect of NaCl on alkaline protease production by *B. licheniformis* N-2 after 24 h incubation at 37 °C and 140 rpm

NaCl conc. (%)	Cell biomass (g/l)	Total protein (mg/ml)	Final pH of the medium	Enzyme activity (PU/ml)
Control	3.42±0.12	3.38±0.04	8.51±0.03	755.02±8.88
0.01	3.52±0.11	3.43±0.08	8.51±0.07	801.22±15.32
0.02	3.57±0.08	3.42±0.03	8.53±0.10	815.08±16.24
0.03	3.68±0.10	3.44±0.05	8.48±0.11	838.18±12.58
0.04	3.81±0.11	3.44±0.07	8.53±0.05	855.81±15.49
0.05	3.84±0.12	3.45±0.05	8.54±0.08	874.61±11.72
0.06	3.84±0.09	3.48±0.06	8.59±0.07	855.35±14.28
0.07	3.80±0.08	3.50±0.08	8.43±0.09	842.19±9.33

control: without external source of NaCl; each value is an average of three parallel replicates ± indicates standard deviation among the replicates

Table 6. Effect of $CaCl_2.2H_2O$ on alkaline protease production by *B. licheniformis* N-2 after 24 h incubation at 37 °C and 140 rpm

$CaCl_2$. $2H_2O$ conc. (%)	Cell biomass (g/l) (gl/l)	Total protein (mg/ml)	Final pH of the medium`	Enzyme activity (PU/ml)
Control	3.02±0.06	3.23±0.11	8.47±0.06	675.22±10.20
0.01	3.18±0.12	3.35±0.12	8.48±0.08	701.28±8.88
0.02	3.36±0.07	3.42±0.13	8.53±0.02	726.17±12.05
0.03	3.62±0.06	3.44±0.08	8.54±0.05	755.71±10.72
0.04	3.78±0.08	3.44±0.10	8.52±0.07	806.45±7.83
0.05	3.84±0.10	3.45±0.09	8.56±0.03	875.05±10.10
0.06	3.91±0.07	3.53±0.05	8.61±0.05	870.12±11.23
0.07	3.88±0.08	3.55±0.06	8.62±0.04	835.43±12.36

control: without external source of $CaCl_2$. $2H_2O$; each value is an average of three parallel replicates ± indicates standard deviation among the replicates

tion at 40 °C without Ca^{2+} ions (Fig. 1). However, least compatibility was determined against Arial up to 7% in the absence of Ca^{2+} ions after 120 min incubation. These findings indicate that addition of suitable stabilizers are essential for proper utilization of alkaline protease against commercial detergents. The enzyme retained more than 50% its stability with most of the detergents in the presence of 5mM of Ca^{2+} ions whereas the maximum stability was determined with Bright Total (72%) followed by Wheel (68.11%) as shown in Fig. 2. After supplementation with 10 mM of Ca^{2+} ions, the enzyme retained more than 65% residual activity against all the detergents even after 120 min incubation at 40 °C (Fig. 3). However, maximum stability about 93.11% was found against Wheel followed by Bright Total (88.34%). Adinarayana *et al.* (2003) reported that alkaline protease retained 65% activity in the presence of Wheel after 3 h of incubation at 60 °C followed by Nirma (58%), Surf Excel (56%) and Ariel (52%). Bhosale *et al*. (1995) reported that protease production by *Conidiobolus coronatus* retained 16 % activity in Revel, 11.4% activity in Ariel and 6.6% activity in Wheel in the presence of 25 mM $CaCl_2.2H_2O$ at 50 °C. Nascimento and Martins (2006)

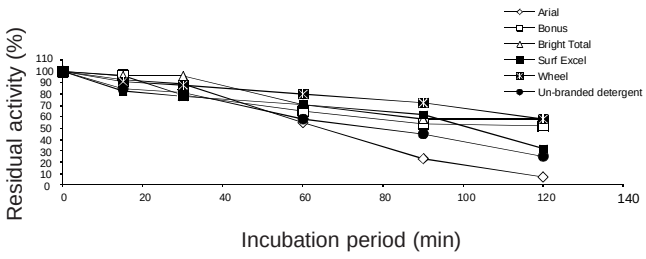

Fig. 1. Compatibility of alkaline ptotease activity from *B. licheniformis* N-2 with different commercial solid detergents. Enzyme activity of control samples (without incubation) was taken as 100%.

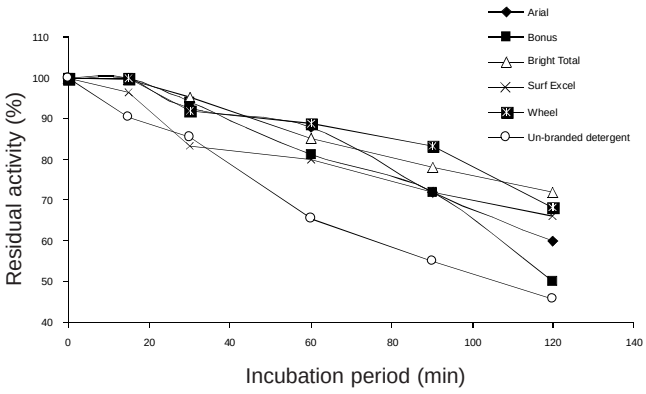

Fig. 2. Compatibility of alkaline protease activity from *B. licheniformis* N-2 with different commercial detergents in the presence of 5 mM $CaCl_2$. $2H_2O$. Enzyme activity of control samples (without incubation) was taken as 100%.

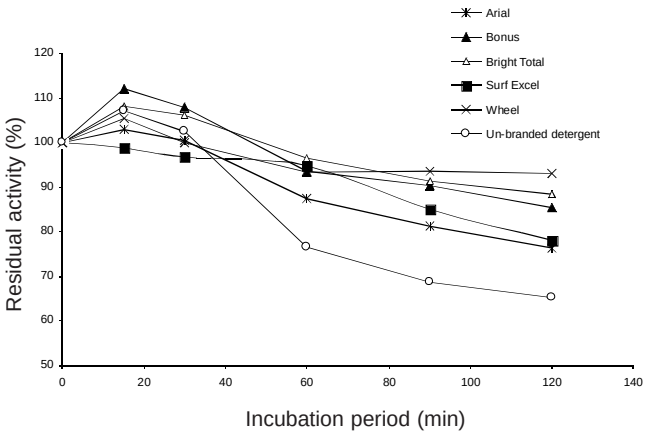

Fig. 3. Compatibility of alkaline protease activity from *B. licheniformis* N-2 with different commercial detergents in the presence of 10 mM $CaCl_2$. $2H_2O$. Enzyme activity of control samples (without incubation) was taken as 100%.

found more than 85% activity in the presence of Ca^{2+} ions after 1 h incubation at 60 °C. Comparing these results, the alkaline protease produced by *B. licheniformis* N-2 was found to be significantly more stable over a broad range of commercial detergents.

Conclusion

Alkaline protease production and growth of *B. licheniformis* N-2 under submerged fermentation were found to be influenced by different concentrations of glucose, soybean meal and various metal ions in the growth medium. The maximum protease production (875.05 PU/ml) was achieved by employing optimized concentrations of glucose (1%), soybean meal (1%), K_2HPO_4 (0.5%), $MgSO_4.7H_2O$ (0.05 %), NaCl (0.05%), $CaCl_2.2H_2O$ (0.05%) after 24 h incubation at 140 rpm. The compatibility studies of alkaline protease with different commercial detergents in the presence and absence of Ca^{2+} ions indicated that the *B. licheniformis* N-2 protease is suitable for detergent formulation. More than 65 % stability was observed with various commercial detergents in the presence of 10 mM $CaCl_2.2H_2O$. Collectively, all these results may justify the possibilities of production of alkaline protease by *B. licheniformis* N-2 for its commercial exploitation in detergent industry.

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Antidiarrhoeal Evaluation of Some Nigerian Medicinal Plants Used in Bini Traditional Folk Medicine

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Abstract. Four medicinal plants namely; *Vernonia amygdalina, Psidium guajava, Chromolaena odorata* and *Anarcadium occidentale*, commonly used for the treatment of diarrhoea in Bini traditional folk medicine in Nigeria were tested against *Escherichia coli, Staphylococcus aureus* and *Klebsiella aerogenes*. The leaf extracts of *P. guajava* and *A. occidentale* completely inhibited the growth of all the organisms tested, while *V. amygdalina* inhibited the growth of *K. aerogenes* only. Metronidazole was used as the standard antidiarrhoeal drug. Glycosides were found in all the plant extracts. This study, favours the use of the leaf extracts of *A. occidentale, P. guajava* and *V. amygdalina* for the treatment of diarrhoea in Nigeria.

Keywords: antidiarrhoeal drugs, medicinal plants, Nigeria, Bini folk medicine

Introduction

Nowadays, the value of medicinal herbs in combating diseases is being rediscovered and the herbal medicine trade has become a booming business worldwide. In India, for example, there are 46,000 licensed pharmacies manufacturing traditional remedies, 80% of which come from plants (Alok, 1991). In Africa, many plant species are reported to have medicinal value (George and Pamplona-Roger, 1997). These plant species are used for simple or complex pathological complications to psychological and mental illnesses.

Gastrointestinal disorders are one of the major health problems in developing countries and generally, plants are used in the treatment of diseases, indigenously. In sub-Saharan Africa in 1999, there were 8, 181 deaths per day due to diarrhoea and traditional medicines were often the only affordable and accessible form of healthcare for the majority of this rural African population (Obuekwe and Obuekwe, 2002). Frequently, tannin-containing plants are used to treat diarrhoea. The bark and leaves of cashew (*Anarcadium occidentale*) are a rich source of tannins–a group pf phytochemicals with documented biological activities. Cashew fruit has exhibited antibacterial activity against the Gram- negative bacterium *Helicobacter pylori*, now considered to be the cause of acute gastritis and stomach ulcers (Heirrich *et al.*, 1992). Its effectiveness against leishmanial ulcers has also been documented in the same report. An infusion of the bark and leaves is an astringent and a mouthwash, used in toothache and is given internally in dysentery (Database entry for Cajueiro -*A. occidentale*). The natural rainforest remedy for diarrhoea is standard decoction of leaves and twigs of *A. occidentale* (Indian Mecicinal Plants- *A. occidentale,* 2001). The antiulcerogenic effect acute, subacute toxicity as well as the genotoxic effect of a hydro-ethanolic extract of the cashew (*A. occidentale* L.) leaves have also been investigated (Konan and Bacchi, 2007; Konan *et al.*, 2007).

Psidium guajava (guava) is a common shade tree or shrub in the tropics. The Tikuna Indians decoct the leaves as a cure for diarrhoea (People and Plants online - working paper 1: African Medicinal Plants). Much of its therapeutic activity is attributed to the flavonoids, which have demonstrated some antibacterial activity. Quercetin is thought to contribute to the antidiarrhoeal effect of guava. It is able to relax intestinal smooth muscles and inhibit bowel contractions. The effective use of guava in diarrhoea, dysentery and gastroenteritis can also be related to guava's documented antibacterial properties. Bark and leaf extracts of the tree have exhibited *in-vitro* toxic action against numerous bacteria (Theunissen, 2002).

A range of medicinal plants with antidiarrhoeal properties have been widely used; but the effectiveness of many of the antidiarrhoeal traditional medicines have not been scientifically evaluated. This study investigates the potential antidiarrhoeal properties of the leaf extracts of four Nigerian medicinal plants *P. guajava, A. occidentale, V. amygdalina* and *C. odorata* against *E. coli*, *S. aureus* and *K. aerogenes*.

Fresh leaves of the plants. *P. guajava, A. occidentale, V. amygdalina* and *C. odorata* were collected locally from Benin City, Nigeria.

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Preparation of extracts for microbiological analysis. Decoctions of the fresh leaves of the plants were made by pounding 1 g of the leaf using sterile mortar and pestle and extracting in 200 ml distilled water to get 5 mg/ml concentration. One ml of each decoction was pipetted and used for *in vitro* agar diffusion assay. Fresh leaves of the plants were also dried in an oven at a temperature of 40 °C for two days. The leaves were ground into fine powder and 150 g of the powdered leaves of the plants were extracted with 2.5 l of ethanol using Soxhlet extractor and concentrated using rotary evaporator.

Cultures of *E. coli, S. aureus* and *K. aerogenes* were obtained as clinical isolates from a reference hospital in Benin City, Nigeria. Overnight cultures of the organisms were obtained by sub-culturing into test tubes containing 10 ml of sterile nutrient broth (Oxoid). Viable counts of the organisms using the standard plate method were 2.4×10^3, 2.16×10^4 and 2.8×10^3 for *E. coli, S. aureus* and *K. aerogenes*, respectively. Using agar diffusion method, 100, 150, 200 and 250 mg/ml concentrations of the plant extracts were pipetted onto plates containing 1 ml of overnight cultures of the test organisms. Overnight cultures were used to ensure that the organisms were at their maximum growth. The strains were maintained on nutrient agar slants and stored in the refrigerator and transferred to fresh slants every two weeks. Cultures were usually transferred to fresh medium before use.

Results were recorded after 24, 48 and 72 h. Metronidazole was used as the standard drug in concentrations 100, 150, 200 and 250 mg/ml. Triplicate tests were made and mean was calculated. The minimum inhibitory concentrations (MICs) for various extracts active against the test organisms were determined using the double strength dilution method.

Phytochemical tests. Phytochemical analyses of the plant extracts were assayed to determine the active chemical compounds. For glycosides, 0.1 g of powdered dried leaves of *A. occidentale* was added to 20 ml distilled water in a beaker. This was boiled gently for 2-3 min, filtered hot and cooled. One ml of the filtrate was tested with Fehling's solutions A and B and the result noted. Five ml of dil. sulphuric acid was added to the remainder of the filtrate and boiled for 3-5 min and later filtered. Filtrate was made slightly alkaline with sodium bicarbonate. Fehling's solutions A and B were added and the mixture boiled. A change in colour from deep blue to green, yellow or red indicated a positive reduction test (red precipitate of cuprous oxide). This test was repeated for *V. amygdalina, P. guajava* and *C. odorata.*

For saponins, 0.1 g of powdered leaves of *A. occidentale* was added to 20 ml distilled water in a beaker and boiled gently for 3 min, filtered hot and cooled. Five ml of the filtrate was shaken vigorously with water and the result was noted. Five ml of dil. sulphuric acid was added to the remainder of the filtrate and boiled gently for 3-5 min, cooled and filtered. Residue was washed into a test tube with 2 ml distilled water, shaken vigorously and the result was recorded. Filtrate was made alkaline with sodium bicarbonate, Fehling's solution was added and boiled. Result was also noted. The test was also repeated for *V. amygdalina, P. guajava* and *C. odorata.*

For tannins, 2 g of powdered leaves of *A. occidentale* was added to 50 ml of distilled water in a beaker, boiled for 5 min, filtered and made up to 50 ml volume. Two drops of test solution and one drop of aqueous ferric chloride were then added to 10 ml distilled water. To 5 ml of test solution, 5 ml of 0.25% ferric ammonium citrate solution and 0.5 g sodium acetate were added, boiled and cooled. A purple/blackish bulky precipitate, insoluble in hot water or blue solution indicated the presence of pseudo tannins. Five ml of the precipitate was further diluted with 10 ml of distilled water and 10 ml of 10% aqueous lead acetate solution was added. Appearance of a precipitate indicated the presence of tannins. These tests were repeated for the other plant materials to be tested.

For flavonoids, 1 g of the powdered leaves of *A. occidentale* was extracted with water by heating on a water bath, cooled and filtered. To 5 ml of the filtrate, 1 ml of sodium hydroxide was added. A yellow solution, which became colourless on addition of concentrated hydrogen chloride, indicated the presence of flavonoids. This was repeated for other plant materials to be tested.

Results and Discussion

Antidiarrhoeal screening of the aqueous extracts of the fresh leaves of four plants showed no activity. After 24 h contact time, 100, 150, 200 and 250 mg/ml of the ethanolic leaf extracts of *A. occidentale, P. guajava* and *V. amygdalina* exhibited activity against the three test organisms; namely, *S. aureus, E. coli* and *K. aerogenes.* The ethanolic leaf extract of *C. odorata* at the same concentrations was active only aginst *K. aerogenes* after 24 h (Table 1).

After 48 h contact time, the ethanolic leaf extracts of only *A. occidentale* and *P. guajava* had activities at the same concentrations as in 24 h contact time. *V. amygdalina* was active at 200 mg/ml concentration, against *S. aureus* only and after 48 h at 250 mg/ml concentration against the 3 test organisms. Further more, after 72 h contact time, the ethanolic leaf extracts of *A. occidentale* and *P. guajava* retained their activities against the 3 test organisms at the same concentrations as after 24 and 48 h contact time. However, with

Table 1. Antibacterial screening of the leaf extracts of four Nigerian medicinal plants

Concentration of ethanolic leaf extracts (mg/ml)	Growth of test organisms								
	K. aerogenes			*E. coli*			*S. aureus*		
	24h	48h	72h	24h	48h	72h	24h	48h	72h
Anarcadium occidentale									
100	-	-	-	-	-	-	-	-	-
150	-	-	-	-	-	-	-	-	-
200	-	-	-	-	-	-	-	-	-
250	-	-	-	-	-	-	-	-	-
Psidium guajava									
100	-	-	-	-	-	-	-	-	-
150	-	-	-	-	-	-	-	-	-
200	-	-	-	-	-	-	-	-	-
250	-	-	-	-	-	-	-	-	-
Vernonia amygdalina									
100	-	+	+	-	+	+	-	+	+
150	-	+	+	-	+	+	-	+	+
200	-	+	+	-	+	+	-	+	+
250	-	-	-	-	-	+	-	-	+
Chromolaena odorata									
100	no activity at all concentrations tested			no activity at all concentrations tested			no activity at all concentrations tested		
150									
200									
250									
Metronidazole (control)									
100	-	-	-	-	-	-	-	-	-
150	-	-	-	-	-	-	-	-	-
200	-	-	-	-	-	-	-	-	-
250	-	-	-	-	-	-	-	-	-

- = no growth (total inhibition of test organism); + = growth (no inhibition)

V. amygdalina, the activity was recorded against only *K. aerogenes* after 72 h contact time and none against *E. coli* and *S. aureus* (Table 1).

Phytochemical screening of the leaf extracts revealed the presence of glycosides and tannins in *A. occidentale*; glycosides, saponins, tannins and flavonoids in *P. guajava*; glycosides, tannins and saponins in *V. amygdalina* and glycosides, tannins and flavonoids in *C. odorata* (Table 2).

The minimum inhibitory concentrations (MICs) of the leaf extracts of *A. occidentale* having antidiarrhoeal activities were 40.02 mg/ml; 22.84 mg/ml and 46.69 mg/ml, those of *P. guajava* were 33.35 mg/ml, 39.97 mg/ml and 53.36 mg/ml, against *K. aerogenes*, *E. coli* and *S. aureus*, respectively, whereas, leaf extract of *V. amygdalina* was active against only *K. areogenes*, in the concentration of 66.67 mg/ml.

Metronidazole was used as the standard antidiarrhoeal drug with MIC of 0.67 mg/ml; 0.571 mg/ml and 0.67 mg/ml for *K. aerogenes*, *E. coli* and *S. aureus*, respectively (Table 3).

Table 2. Phytochemical analysis of the ethanolic extract of four medicinal plants

Plant	Compounds found
Psidium guajava	glycosides, saponins, tannins and flavonoids
Anarcadium occidentale	glycosides and tannins
Vernonia amygdalina	glycosides, tannins and saponins
Chromolaena odorata	glycosides, tannins and flavonoids

The results of this study show that the leaf extract of *A. occidentale* (cashew) possesses antidiarhoeal activity. This correlates with works of other investigators (Database entry for Cajueiro - *Anarcadium occidentale*; Indian Medicinal Plants, *Anarcadium occidentale*, 2001; Konan and Bacchi, 2007; Akinpelu, 2001). Today in Peruvian herbal medicine, cashew leaf tea called 'casho' is employed as a common diarrhoeal remedy. Methanolic bark extract of *A. occidentale* has also been found to exhibit *in vitro* antimicrobial activity

Table 3. Minimum inhibitory concentrations (MICs) of the plant extracts having antidiarrhoeal activities

Plant extracts (mg/ml)	*K. aerogenes*	*E. coli*	*S. aureus*
Anarcadium occidentale	40.02	22.84	46.69
Psidium guajava	33.35	39.97	53.36
Vernonia amygdalina	66.67	-	-
Metrinadizole (standard drug used as control)	0.67	0.571	0.67

against 13 of 15 microorganisms tested at a concentration of 20 mg/ml (Akinpelu, 2001). An essential oil obtained by steam distillation of the leaves of *A. occidentale* acted as a tranquilizer and analgesic and had mild antibacterial and antifungal activities (Indian Medicinal Plants: *Anarcadium occidentale*, 2001).

Psidium guajava also possesses antidiarrhoeal activities. Its bark and leaf extracts have been found to possess *in vitro* toxic action against numerous bacteria (People and Plants online-working paper1: African Medicinal Plants). In several studies, guava had shown significant antibacterial activity against such common diarrhoea-causing bacteria as *Staphylococcus*, *Shigella*, *Bacillus*, *Salmonella*, *Escherichia coli*, *Clostridium* and *Pseudomona*s (Theunissen, 2002). The effectiveness of *P. guajava* as an antidiarrhoeal agent has been confirmed; the methanolic extract is the only agent showing significant inhibitory activity against the growth of *Salmonella* and *Shigella* species and enteropathogenic *E. coli* (Caceres *et al*., 1993).

In this study, *V. amygdalina* showed antidiarrhoeal activity on *K. aerogenes* at 250 mg/ml after 72 h contact time and not on the other test organisms even though antidiarrhoeal activity of the plant has been indicated in the reports of other investigators (Akinpelu, 1999; Obaseiki-Ebor *et al*., 1993). Phytochemical analyses of the leaf extracts revealed the presence of glycosides, tannins, saponins and flavonoids. Medicinal plants had earlier been screened for groups of phytochemical compounds with antibacterial and antiamoebic activities and were found to contain tannins, alkaloids, saponins, flavonoids, steroids and/or triterpenes and reducing sugars (Otshudi *et al*., 2000).

The search for new active chemical compounds in high biological diversity regions has become a challenge to modern pharmaceutical industries. The history of drug discovery implies that the ethnobotanical approach is the most productive of plant surveying methods (Cox and Balick, 1994). The antiulcerogenic effect and acute toxicity of hydroethanolic extract from the cashew (*A. occidentale* L.) leaves have been investigated by Konan and Bacchi (2007) and the extract was found to inhibit gastric lesions induced by HCl/ethanol in female rats and its a methanolic fraction (257.12 mg/kg) reduced gastric lesions by 88.20%. Thus the plant is likely to contain the active principle with antiulcer effect.

In conclusion, the ethanolic extracts of the medicinal plants investigated in this study were found to possess good antidiarrhoeal potential and supported the claims made in Bini folklore medicine. The study suggests that the use of an effective and economical herbal formulation based on such extracts could be beneficial against certain bacterial infections.

Further studies on these plants are required to identify and isolate the specific glycosides that are responsible for the antidiarrhoeal activities with a view to producing new antidiarrhoeal drugs from them.

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Short Communication

Proximate Composition of Head of Wild and Farmed *Catla catla*

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Abstract. In the study for finding out the proximate composition of head of wild and farmed *Catla catla*, the moisture contents were recorded as 63.06 ± 0.46% and 54.91 ± 0.53%, protein contents as 14.77 ± 0.37% and 19.92 ± 0.44%, lipids contents as 7.56 ± 0.46% and 11.90 ± 0.25%, ash contents as 12.26 ± 0.52% and 12.40 ± 0.31% and total nitrogen free extract as 2.31 ± 0.05% and 0.74 ± 0.03%, respectively. Thus farmed fish contained more lipid and protein contents as compared to that of wild fish.

Keywords: *Catla catla*, head compositon, farmed fish

Aquaculture is a low energy expenditure and protein yielding in comparison to other agriculture sectors. Fish has long been recognized as high quality of food for human consumption.

Fish lipids have great nutritional significance owing to their protective role against the cardiovascular diseases as fish is rich in unsaturated fatty acids. Moreover, the lipids provide energy 9.3 cal/g twice than that of protein (McGraw Hill, 1977). Fish has attainded great nutritional significance, in recent years, as the best source of proteins and healthy oils. Farmed *Labeo rohita* possesses higher nutritional as well as commercial value as compared to that of wild fish (Mahboob *et al.*, 2004).

During dressing of fish, head, fins, scales and skin are discarded which, with better management, can be put to better use with economic benefits (Choi and Regenstein, 2000). Fish skin and scales that are discarded as dressing losses are an important source of protein, lipids and minerals (Iqbal, 2002).

Fish collagen in general has more amino acid contents than mammalian collagen (Grossman and Bergman, 1992). Extraction of gelatin has been reported from cod (Gudmunsson and Hafsteinsson, 1997), Tilapia (Grossman and Bergman, 1992), shark skin, lung fish skin, carp skin (absent in the reference). Jamilah and Harvinder (2002) extracted gelatin from the skin of black and red Tilapia and determined the physiochemical characteristics. *Catla catla* (local common names: Taylee, Theila) is one of the major fresh water carps, native to the subcontinent Indo-Pak and neighboring countries of Bangladesh, Nepal and Myanmar. It is the 2nd most important species of the Rohu, consumed by the people, and an important contributer to the aquaculture production. According to (Choi and Regenstein, 2000) the discarded portions of fish such as head, fins, skin, scales can be used for poultry feedings. Head is used as food in different parts of the world but mainly it is lost during dressing.

Present study is an effort towards determining the composition of the wild and farmed varieties of *Catla catla*. The study is based on 21 farmed and 21 wild *Catla catla* of three different weight categories, (450-800 g), (850-1200 g) and (1250-1600 g), procured from Fish Hatchery Satiana Road, Faisalabad and Head Trimu, respectively, which were transported live to Fisheries Research Laboratory, of GC University (Faisalabad Pakistan) for the analysis.

After washing and dissecting the selected heads were weighed on electrical balance. The samples were then minced and immediately oven dried (65-70 °C) for 24 h on less than 100 mg/Hg to determine moisture contents (loss in weight was calculated as moisture percentage). Total nitrogen was determined by the automatic analyzer made by Tecator of Sweden, based on Kjeldahl's method. Fat contents were determined by Soxhlet apparatus.

Fish head normally contains only traces of nitrogen free extract (carbohydrates) in the form of sugars, sugar phosphate and glycogen. Total carbohydrates (%) were estimated as nitrogen free extract by subtracting the total amount of proteins, lipids, ash and water.

Ash contents were calculated as percentage of the weight of the sample afer ashing it at 450 °C for 12 h until while ash was obtained.

The results are summarised in Table 1. The moisture content in head of the wild *Catla catla* was recorded as 63.06 ± 0.46 percent and in farmed fish, were as 54.91± 0.53. The wild variety had the highest moisture contents. Protein contents of wild and formed *C. catla* were 14.77 ± 0.37% and 19.92 ± 0.44%,

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respectively. Statistical analysis showed that farmed *C. catla* gave the maximum protein contents. These results are in line with Srikanth *et al*. (1989) who reported that moisture contents were lowest and protein deposition, highest under the influence of fertilizer treatment in *Cyprinus carpio*. Decrease in protein contents with an increase in body weight are in agreement with the results of Al-Asgah (1992) who reported the crude protein contents had decreasing trend with the increase in weight and size of *Oreochromis niloticus*. Mahboob *et al*. (2004) also found higher protein content in the farmed *Labeo rohita* than that in the wild species.

Table 1. Proximate composition of the head of *C. catla*, wild and farmed varieties (%)

Parameter	*Catla catla*	
(Mean ± SE)	Wild fish	Farmed fish
Moisture	63.06±0.46	54.91±0.53
Protein	14.77±0.37	19.92±0.44
Lipids	7.56±0.46	11.90±0.25
Ash	12.26±0.52	12.40±0.31
Total nitrogen free extract	2.31±0.05	0.74±0.03

In the head of wild and formed *C. catla*, lipids contents were measured as 7.56 ± 0.46% and 11.90 ± 0.25%, respectively. The marked difference in the protein and fat contents of wild fish seem to be due to scarcity of food and the resulting decreased growth. Fish showed progressive reduction in fat resources, yet before reaching a critical low level, proteins began to be utilized for energetic purposes and ultimately a reduction occured in the protein contents with increased water contents. Hassan, 1996). The fish at first consume lipids from liver and starts to mobilize muscle proteins only when fat-derived-energy is nearly used up. After that, as protein is utilized, water moves in to take its place. Such a shift results in the increased water contents that are inversely correlated with protein and fat reserves of their meats (Shimma and Sato, 1985; Mahboob and Sheri, 1997).

Total ash contents in wild and farmed *C. catla* were 12.26 ± 0.52% and 12.40 ± 0.31%, respectively, with little difference between them. Total nitrogen free extract in head of wild and farmed *C. catla* were recorded as 2.31 ± 0.05% and 0.74 ± 0.03%, respectively, with maximum nitrogen free extract being recorded in head of the wild variety.

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Review

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A Comprehensive Systematic Pharmacological Review on *Harpagophytum procumbens* DC. (Devil's claw)

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Abstract. Popularly known as Devil's claw, *Harpagophytum procumbens* DC. (Pedaliaceae) is native to the Kalahari savannas of southern Africa and Namibia. It has been widely used to treat rheumatism. Its secondary tuberous roots contain iridoid glycosides (procumbide, procumboside, harpagoside) as the active principles. This species seems to stimulate migration of interleukins and leucocytes to painful and inflamed joint areas. The drug is indicated for osteoarthritis, degenerative disease of the joints and arthritic processes. Although, *in vivo* pharmacological studies have been carried out in different animal models, with different methodologies and different types of extracts, producing contradictory results, recent clinical studies have shown that *H. procumbens* could be a valid alternative to conventional drugs, especially in the treatment of lumbar pain.

Keywords: *Harpagophytum procumbens*, osteoarthritis, joint ailments, irridoid glycosides

Introduction

Harpagophytum procumbens DC. (Pedaliaceae), commonly known as Devil's claw, is a perennial herbaceous plant native to the arid steppes. It is virtually restricted to the southern part of the African continent, occurring mainly in South Africa, Namibia and Botswana, where it is known locally not only as Devil's claw, but also as grapple plant, wood spider and harpago.

H. procumbens occurs in areas with low annual rainfall (150-500 mm/year) on deep sandy soils of the Kalahari. It is found in savannah vegetation dominated by *Acacia* sp., but does not compete well with grasses. In fact, *H. procumbens* is most often found in areas where grass cover is less than 25% and where the herb cover is less than 20%. It is most abundant in open, trampled and overgrazed areas (Hachfeld, 2002), where it has clumped distribution (Raimondo and Donaldson, 2002).

In an area between the northern Cape and north west provinces of south Africa, densities were estimated at 50 plants/ha in the dense grasses of a well managed farm, 150 plants/ha on unharvested overgrazed communal land near the village of Madibeng and only 11 plants/ha on harvested communal land near Madibeng (Stewart and Cole, 2005).

H. procumbens is a weedy, tuberous plant with creeping annual stems up to 2 m long. The above-ground stems emerge after the first rains and die back in winter and during droughts. Stems grow from a persistent succulent primary tuber, called "mother tuber" by harvesters, the tap root of which can extend to a depth of 2 m. A number of secondary tubers, called "babies", emanate from the primary tuber via fleshy roots. The secondary tubers are up to 25 cm long and 6 cm thick (Schneider, 1997). The secondary tubers contain up to 46% stachyose, a photosynthetic storage product, which is thought to be an adaptation to drought conditions (Stewart and Cole, 2005).

The leaves are opposite, blue-green and usually have several lobes. The flowers are tubular and deep mauve-pink with yellow and white throats. They are open for one day and are pollinated by bees (Von Willert and Sanders, 2004). The flat woody capsules, which give the plant its scientific and common names, bear two rows of curved appendages studded with curved spines (*Harpagophytum* literally means grapple hook plant). The fruit is dispersed by animals (Ernst *et al*., 1988), as it attaches readily to fur and wool, and is also wind dispersed to some degree, as a breeze can carry a fruit some distance from the parent plant.

Seeds have a high degree of dormancy (Stewart and Cole, 2005; De Jong, 1985), which may be an adaptation to drought (Ernst *et al*., 1988). Ernst *et al*. (1988) estimated that only 20-25% of the seeds in a fruit establish soil contact in a given year, suggesting that this may be an adaptation to animal (or wind) dispersal. The same authors also estimated that seeds may remain viable in the seed bank for more than 20 years, by virtue of their low respiration rate.

The thick, fleshy, tuberous secondary tap roots of *H. procumbens* are usually dried and used in south African traditional medicine.

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Since its introduction to Europe from Africa in the early 1900s, dried tubers of the plant have been used to restore appetite, relieve heartburn and reduce pain and inflammation (Soulimani *et al.*, 1994; Costa De Pasquale *et al*., 1985; Occhiuto *et al*., 1985; Grahame and Robinson, 1981). There is increasing evidence to suggest that Devil's claw tubers may help relieve the pain and inflammation of arthritis and other painful disorders, though the mechanism of action is not yet well understood (Baghdikian *et al*., 1997; Lanhers *et al*., 1992; Whitehouse *et al*., 1983).

H. procumbens secondary root is used for an array of human ailments in the form of decoctions, infusions, tinctures and extracts. It has an ethnomedical reputation for efficacy in anorexia, indigestion, diabetes mellitus, hypertension, gout, fevers, skin cancer, infectious diseases, allergies, osteoarthritis, fibrositis and rheumatism, being particularly effective in small joint diseases.

When taken on a regular daily basis, it has a subtle laxative effect. Small doses of the plant's root extract are used for menstrual cramps, while higher doses help placental expulsion. Devil's claw is also used post partum as an analgesic and to keep the uterus contracted. The dry, powdered tuberous root of the plant is used directly as a wound dressing or mixed with animal fat or vaseline as a wound-healing and burn-healing ointment. Traditional ointments and creams of *H. procumbens* are applied topically for minor muscular aches and pains, and to painful joints. Traditional health practitioners of south Africa have also claimed that *H. procumbens* secondary root extract is useful for the treatment, management and/or control of epilepsy and childhood convulsions.

Extracts of *H. procumbens* are currently the focus of research as a potential therapeutic agent to treat rheumatoid arthritis and pain (Ernest and Chrubasik, 2000; Fox, 2000; Leblan *et al.*, 2000; Chrubasik *et al*., 1999).

There is increasing evidence to suggest that they may help relieve the pain and inflammation of arthritis and other painful disorders, although their mechanism of action (Baghdikian *et al*., 1997; Lanhers *et al*., 1992; Whitehouse *et al*., 1983) is not yet well understood and the active principles have not been identified unequivocally.

In this review we consider the botanical description, ethnopharmacology and phytochemistry of *H. procumbens*. Biological activity, pharmacology and toxicity are discussed and the results of recent clinical studies are presented as a basis for evaluating *H. procumbens* as an adjuvant in the treatment of pain and osteoarthritis.

Chemical constituents. The major class of compounds with therapeutic activity are iridoids, concentrations of which may vary from 0.5 to 3% in the dry tuber. Other parts of the plant (flowers, stems and ripe fruit) contain no iridoids or only traces (leaves) (Capasso *et al*., 2006). The main iridoids in *H. procumbens* are harpagoside, harpagide and procumbide. Harpagoside and its congeners occur as two isomers in mutual equilibrium: an open form with two free aldehyde groups and a cyclic form by virtue of an enol-ether bridge (Fig. 1). The cyclic configuration is stable when the molecule is gylcosylated (Van Haelen *et al.*, 1983). Hydrolysis of iridoids produces genins having a structure similar to that of certain prostaglandins.

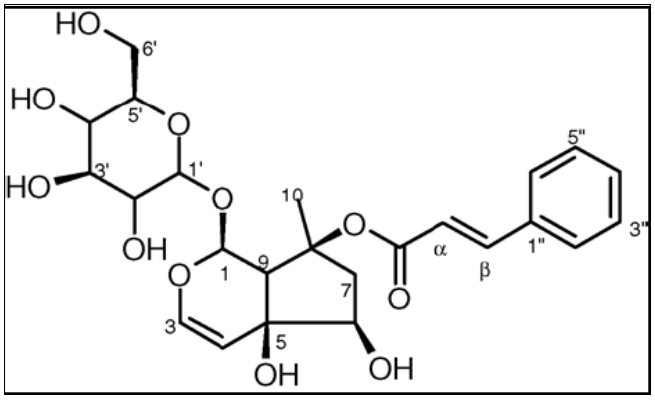

Fig. 1. Structure of harpagoside (8-O-E-cinnamoyl-harpagide).

The species *H. zeyehri*, the secondary roots of which are difficult to distinguish by microscope examination, has a different content and distribution of iridoids. Determination of the ratio of harpagoside and 8-*p*-coumaroyl-harpagide content has been proposed as a unequivocal chemotaxonomic method of differentiating *H. procumbens* from *H. zeyehri* (Baghdikian *et al.*, 1997).

In a recent study a thorough investigation of Devil's claw tubers led to the isolation of eleven iridoid glycosides, two of which were new (Qi *et al*., 2006).

Harpagoside and other iridoids are substances with a bitterness value of up to 120. Limits to bitterness value have not been imposed, but a minimum of 1.8% of amaroids is sufficiently high. Standardisation of extracts of the drug is based on harpagoside concentration (Bisset and Wichtl, 2001).

Besides iridoids, phytochemical studies on secondary roots of grapple plant have revealed a quinone (harpagoquinone), some aromatic acids (cinnamic acid and chlorogenic acid) and some flavonoids (kaempferol, kaempferide, fisetine and luteoline). Pentacyclic triterpenic acids (oleanolic acid and ursolic acid) and small quantities of a resin and an essential oil have also been found. Sterols, fats, hydrocarbons and

holosides have also been detected (Van Haelen, 1986). Two new phenolic glycosides (acteoside and isoacteoside) were identified by Burger *et al.* (1987). Major quantities of carbohydrates (stachyose) and several tetrahalosides bearing glucose and sucrose molecules have also been found. The presence of stachyose indicates a chemotaxonomic relationship of the Pedaliaceae family with the Lamiaceae and Verbenaceae (Van Haelen, 1986).

Pharmacology. Commercial preparations of Harpago contain 1.4-2.0% of harpagoside. The results of research into this species are difficult to compare and interpret, mainly because of the variety of extracts used. Methanolic extract and aqueous extract were the most common but differed in harpagoside content.

The World Health Organisation (WHO, 2007) lists Devil's claw for rheumatic pain, loss of appetite and dyspepsia and as adjuvant for degenerative disease of the locomotor system.

In vitro experiments. Recent *in vitro* studies, well listed in the review published by Grant *et al*. (2007), indicate that grapple plant preparations may interact with the inflammation cascade, including synthesis and activity of cytokines. Aqueous extract has been found to suppress expression of cyclooxygenase-2 (COX2), stimulated by lipopolysaccharides (LPS) and nitric oxide synthetase, in murine L929 fibroblasts. Lipopolysaccharides trigger a wide series of cell responses that play a major role in the pathogenesis of inflammatory reactions, including activation of inflammatory cells and production of cytokines and other mediators. Prostaglandin E2, synthesized by arachidonic acid via the cyclooxygenase enzyme complex, is a key mediator. The cyclooxygenase-1 (COX1) isoform is constitutional whereas COX2 is only expressed in response to proinflammatory signals, such as those of cytokines and bacterial endotoxin LPS. COX2 produces a large quantity of prostaglandin E2 which causes inflammation (Mitchell *et al*., 1995).

Nitric oxide (NO), synthesized from L-arginine by NO-synthetase (NOS), plays a major role in the regulation of many physiological processes. The various isoforms of NOS can be classified as inducible (iNOS), endothelial (eNOS) and neuronal NOS (nNOS). The first of the three is involved in inflammatory phenomena.

The anti-inflammatory and analgesic effect of aqueous extract of Devil's calw was recently confirmed (Jang *et al*., 2003) by assessing cell viability (MTT test) and expression of COX2 and iNOS (quantity of nitrite in cell supernatant) by reverse transcription polymerase chain reaction (RT-PCR) in L929 fibroblasts stimulated with LPS. The suppression of COX2 and iNOS expression found inhibited prostaglandin E2 synthesis.

Another study by Kaszkin *et al*. (2004) evaluated the utility of Harpago extract in the treatment of inflammatory kidney disease. Two extracts containing 8.9% (ex1) and 27% (ex2) harpagoside were tested for effects on IL-1-induced production of NO and on regulation of transcription of iNOS in rat renal cells. An 80% dose-dependent reduction in the formation of NO, due to inhibition of iNOS expression, was observed. A reduction in activity of the iNOS promotor of nuclear translocation of NF-kB was also found, indicating that the extracts impaired transcriptional activation of iNOS. This effect was confirmed by Huang *et al*. (2006) who demonstrated inhibition of mRNA levels induced by LPS and COX2 and iNOS expression in human hepatocarcinoma cells. Harpagoside also blocked activity of the NF-kB promoter stimulated by LPS in a dose-dependent manner in RAW264.7 cells. The results of the study show that inhibition of COX2 and iNOS expression by harpagoside involves suppression of NF-kP activation, resulting in inhibition of the inflammatory process and associated pain.

Other extracts containing lower concentrations of harpagoside did not cause this inhibition. Harpagoside alone only works at concentrations in the range 0.3-1.0 mg/ml which are much higher than those of total extracts, and a preparation completely devoid of the molecule strongly inhibited iNOS expression, indicating that other constituents are also involved in its activity. An extract also had a strong antioxidant effect which harpagoside does not have (Rindone, 2006).

A recent study investigated the mechanism of action of harpagoside, using human HepG2 hepatocarcinoma and RAW 264.7 macrophage cell lines. Harpagoside inhibited LPS-induced mRNA levels and protein expression of COX2 and inducible nitric oxide in HepG2 cells. These inhibitions appeared correlated with suppression of NF-kB activation by harpagoside, suggesting that inhibition of expression of COX2 and iNOS by harpagoside involves suppression of NF-kB activation, thereby inhibiting downstream inflammation and pain (Huang *et al*., 2006). These results show that certain extracts of Devil's claw could be an interesting source of drugs for treating glomerular and other inflammatory diseases.

Other *in vitro* studies have shown a significant decrease in synthesis of cell membrane degradation enzymes in isolated chondrocytes and dose-dependent inhibition of elastase. Inflammatory diseases of the joints, such as rheumatoid arthritis and osteoarthritis are characterised by loss of joint cartilage related to an imbalance between synthesis and breakdown of

the cartilagionous extracellular matrix. These diseases are accompanied by elevated induction of cytokines such as IL-1b and TNF-a. Increased release of cytokines leads to increased production of degradation enzymes, such as metalloproteases (MMPs). A significant reduction in MMP synthesis was recently demonstrated in human chondrocytes stimulated with IL-1b. Two extracts were used. The first contained 210 mg and the other 480 mg of dry extract; the latter was more effective (Schulze-Tanzil *et al*., 2004). The effect of Devil's claw on arthritis could be due to its capacity to suppress MMP production by inhibiting synthesis of proinflammatory cytokines.

As far as leucocyte elastase is concerned, aqueous extract showed rather weak, dose-dependent inhibitory activity (Boje *et al*., 2003).

A study by Fiebich *et al*. (2001) on *H. procumbens* extract SteiHap 69 (Steiner *Harpagophyton procumbens* extract 69) demonstrated its close-dependent anti-inflammatory effects by preventing the LPS-induced synthesis of tumour-necrosis factor α (TNF∗) by human monocytes. However, harpagide and harpagoside had no effect on LPS-induced TNF∗ release.

In vivo experiments. *Anti-inflammatory and analgesic activity*. At first sight, the results of the few pharmacological studies conducted on *H. procumbens* seem contradictory, since they were based on different methods and on different animal models (acute and subacute inflammation) with different extracts administered by different routes (Bruneton, 2002). Recent results show that aqueous extract administered intraperitoneally at doses from 100 to 400 mg/kg is active in a dose-dependent manner on carrageenan-induced edema in rat paws. The same extract was inactive when administered orally, presumably due to gastric breakdown of the active principle. The extract was also inactive when given parenterally if previously treated with acids. It was active when administered intra-duodenally.

Under the same experimental conditions, harpagoside was inactive, but was found to promote peripheral analgesic activity of the aqueous extract (100 mg/kg i.p.). The authors suggested research into formulations that could improve the bioavailability of oral harpagoside. In a pilot study, plasma concentrations of 4 ng/ml and 15 ng/ml harpagoside were found 15 min and 2 h, respectively, after administration of 600 mg extract containing 50 mg harpagoside (Loew *et al*., 2001). However, the role of harpagoside remains controversial. Oral administration of aqueous extract of *Scrophularia frutescens* DC. (Scrophularieceae) to rats is reported to have antiinflammatory activity on carrageenan-induced edema that does not seem related to harpagoside (Garciá *et al*., 1996).

Adjuvant-induced arthritis is often used as a model of chronic and subchronic inflammation and is important in the study of pharmacological control of inflammatory proceses and in assessing the antiinflammatory and analgesic properties of drugs. One reason for the widespread use of this model has to do with the strong correlation between efficacy of drugs in this model and efficacy in humans (rheumatoid arthritis). In a study aimed at investigating the antiinflammatory effects of *H. procumbens*, Andersen *et al*. (2004) induced arthritis by acute and chronic treatment. A single administration of three doses of extract (25, 50 and 100 mg/kg) had some analgesic effect, measured on the basis of response to thermal stimuli (hot plate test). The same effect was observed after chronic administration.

Kundu *et al*. (2005) reported a study of the antiinflammatory mechanism of Devil's claw. Methanol extract was tested for effects on expression and COX2 induced by TPA (a phorbol ester) in mouse skin. Topical application of the extract inhibited directly-induced COX2 expression, decreasing the catalytic activity of extracellular signal-regulated protein kinase (ERK), which regulates activation of transcription factors that mediate COX2 induction. The extract inhibited activation of activator protein-1 (AP1) and attenuated expression of its key component c-Fos, while nuclear factor NFB was unaffected (Kundu *et al*., 2005).

Another mechanism of action study (Na *et al*., 2004) assessed inhibition of COX2 expression induced by TPA in human mammary epithelial cells MCF10A and on mouse skin *in vivo*. Methanol extract of devil's claw inhibited binding of NFB transcription factor to DNA in MCF10A cells in a dose-dependent way. Suppression of DNA binding suggests a chemopreventive effect.

In a study, Mahomed and Ojewole (2004) evaluated the analgesic effect (hot plate and acetic acid test), antiinflammatory effect (albumin-induced edema) and antidiabetic effect (streptozotocine-induced diabetes) of aqueous extract in rats. Diclofenac (100 mg/kg i.p.) and clorpropamide were used as reference drugs. Aqueous extract (50-800 mg/kg i.p.) had a significant analgesic effect on nociceptive thermal and chemical stimulation in mice and a significant dose-dependent reduction in albumin-induced edema. The extract also caused a reduction in plasma levels of glucose in normal rats and rats with streptozotocin-induced edema. These results seem to explain popular use of this plant as an analgesic and to alleviate inflammation and diabetes in south Africa.

Clinical studies. There have not been many clinical tests on humans and their heterogeneity makes it difficult to draw conclusions. It has been demonstrated that grapple plant

alleviates pain in chronic inflammation of the joints but its efficacy seems less than that of aspirin and indomethacin (Newall *et al*., 1996). However, certain preparations of the drug and special physical exercises can be a valid alternative to synthetic drugs, especially in the treatment of lumbago.

The results of studies on *H. procumbens* are not easy to compare and interpret, mainly because of the variety of extracts used. Methanol and aqueous extracts are, however, the most widely used in these studies. In Europe, Doloteffin, extract WS 1531® and extract LI174® are used. The preparations differ in their harpagoside content.

Since *H. procumbens* has a broader mechanism of action that conventional NSAIDs, inhibiting both cyclooxygenases and lipooxygenases involved in the metabolic pathway of arachidonic acid, it has been tested clinically in certain rheumatic inflammatory diseases.

The primary end-points of the clinical trials conducted on *H. procumbens* were a reduction of inflammation and pain, as well as improved movement and mobility of patients with rheumatism. As shown by the examples that follow, the plant proved to be just as effective in reducing these parameters as certain conventional treatments with which it was compared. To assess the efficacy of *H. procumbens* in the treatment of lumbago and osteoarthritis, it is worth considering the systematic review of clinical studies by Grant *et al*. (2007), by Setty and Sigal (2005), and by Gagnier *et al*. (2004).

Searches in PubMed, Embase, Cochrane Controlled Trials Registry, Cochrane Musculoskeletal Specialized Register, Dissertation Abstracts, BIDS ISI and Cochrane Complementary Medicine Fields Specialized Register brought to light 130 references to clinical studies on *H. procumbens*. After exclusion of duplicates and articles based on inadequate qualitative criteria, 12 randomized studies were selected, four on the treatment of lumbar pain (Chrubasik *et al*., 2003, 1999, 1996), five on the treatment of osteoarthritis (Biller, 2002; Frerik *et al*., 2001; Chantre *et al*., 2000; Lecomte and Costa, 1992; Schruffler, 1980) and three on the treatment of various other forms of pain involving muscles and bones (Pugno, 2006).

Of the four studies on treatment of lumbago (total number of patients 505), two compare *H. procumbens* with placebo, one with various conventional therapies (e.g., antiinflammatory drugs, massage, physical exercise) and one with COX2 inhibitor, rofecoxib. Of the five studies on treatment of osteoarthritis (total number of patients 385), three compare the plant with placebo and two with standard drugs. All three studies on treatment of other forms of muscle and bone pain compared *H. procumbens* with placebo.

A recent study examined, whether the anti-inflammatory response to whole extract of *H. procumbens* in rats was a consequence of adrenal corticosteroid release. Carrageenan-induced inflammatory response in the hindpaws was evaluated in control, sham-operated and adrenalectomized rats. The extract was administered orally (by gavage) or intraperitoneally, 30 min prior to nociceptive stimulus. Blood samples were then collected, and the number of circulating leukocytes was estimated. The results showed that whole extract of *H. procumbens* administered intraperitoneally had an inhibitory effect on acute inflammatory response, irrespective of the participation of adrenal corticosteroids. When administered orally, the extract was ineffective (Catelan *et al*., 2006).

Lumbar pain. Chrubasik *et al*. (1997) treated 102 lumbago patients with 4500 mg/day of aqueous extract of *H. procumbens* (30 mg/day harpagoside) or with conventional NSAIDs or with physical manipulation, for 6 weeks. No significant differences in pain-free time and change in Arthus index were found between patients undergoing the three treatments.

Chrubasik *et al*. (1999) subsequently treated 197 patients with pseudoradiating and non radiating lumbago with 4500 mg/day of aqueous extract of dried tubers of *H. procumbens* (50 mg/day harpagoside), with 9000 mg/day of the same extract (100 mg/day harpagoside) or with placebo. Patients treated with either dose of extract showed an improvement in pain with respect to patients treated with placebo.

The next prospective, randomized, double-blind study by Chrubasik *et al*. (2003) was particularly interesting, because it compared the effects of commercial aqueous extract of dessicated, pulverized tubers with that of the latest generation synthetic antiinflammatory agent, rofecoxib, a selective COX2 inhibitor. The subjects were 88 patients, age 45-75 years, with at least a six-month history of rheumatic back pain, worsening in the 8 weeks prior to the study, divided into two groups of 44 patients. The first group received 2400 mg/day of extract (60 mg/day harpagoside) for 6 weeks plus one tablet/day of placebo. The second group received a tablet per day of rofecoxib (12.5 mg) and two tablets of placebo three times a day. Patients of both groups could also take up to 400 mg/day tramadol drops (2.5 mg/ml), a synthetic opioid receptor agonist.

During treatment, patients kept a diary in which they scored pain intensity on a 5 point scale (no pain, slight, moderate, strong and acute pain) and the daily dose of tramadol taken. Patients also answered a standardised questionnaire on general health, daily activities and type of pain, a questionnaire on depression (Beck Depression Inventory BDI) and a health assessment questionnaire (HAQ).

Forty-three patients in the *H. procumbens* group and 36 in the rofecoxib group completed treatment. The number of patients responding completely to therapy increased up to the fifth week, more or less in the same manner in both groups. At week 6, the total number of responses reached 17% of patients (10 patients in the *H. procumbens* group and five patients in the rofecoxib group). Comparison of the weekly mean pain scores between weeks four and six did not show significant differences in the number of patients showing 20-50% improvement in the two groups. The Arthus pain index decreased by about 10% during treatment. The greatest reduction was recorded in week 2.

Thirty-four of the 88 patients (38.6%) resorted to tramadol, 21 in the *H. procumbens* group and 13 in the rofecoxib group. Mean consumption of tramadol over 6 weeks was 230 mg in the first and 133 mg in the second group.

Fourteen patients reported side-effects on 39 occasions, 28 of which (13 in the *H. procumbens* group) could be ascribed to treatment. Gastrointestinal disorders were reported by 8 patients in the *H. procumbens* group and 9 in the rofecoxib group; in the latter, gastrointestinal effects were more severe, causing 5 patients to drop out.

Analysis of the results did not reveal any statistically significant differences in effect on lumbar pain between the two therapies. The study was completed with follow-up one year later (Chrubasik *et al*., 2005). Thirty-eight patients previously in the *H. procumbens* group and 35 in the rofecoxib group were treated daily up to 54 weeks with a dose of extract containing 60 mg harpagoside. Fifty-three patients completed 24 weeks of follow-up and 43 completed 54 weeks. No significant difference in daily pain score, resort to other analgesics, Arthus index and HAQ score was found between patients previously in the two groups. Apart from individual fluctuations, follow-up showed a slight improvement in Arthus index and HAQ with respect to the initial study. On the basis of 21,761 patient days, the percentages of patients who were pain-free, or with slight, moderate, strong or acute pain were 28, 39, 22, 8.5 and 1.5%, respectively. Three patients reported minor side-effects.

The authors concluded that maintenance therapy with *H. procumbens* in patients previously treated with the same extract or with rofecoxib produced similar effects and that long-term treatment with the extract was well tolerated.

Osteoarthritis. In 1980, Schrulffer treated 50 osteoarthritis patients with 2500 mg/day of aqueous extract of *H. procumbens* (less than 30 mg/day harpagoside) or with phenylbutazone for 30 days. Grapple plant was found to attenuate pain and improve physical condition better than phenylbutazone.

Lecomte and Costa (1992) treated 89 patients with arthritis of different joints (spine, neck, hip, knee) with powdered secondary root of *H. procumbens* (200 mg/day equivalent to 60 mg/day harpagoside) or placebo for 60 days. The Schober test showed that pain improved more in patients receiving *H. procumbens* than in those receiving placebo.

Chantre *et al*. (2000) treated 122 osteoarthritis patients with hip or knee pain with 4500 mg/day of powder of cold-dried secondary roots of grapple (equivalent to 57 mg/day harpagoside) or with the NSAID diacerein, also known as diacetylrhein, for 16 weeks. Intent-to-treat analysis showed that the plant was not less effective than the drug.

In 2001, Frerik *et al*., treated 46 osteoarthritis patients with 4500 mg/day hydroethanolic extract (Teufelskrallenextrakt LoHar 45) (60% ethanol) of *H. procumbens* (equivalent to less than 30 mg/day harpagoside) or placebo for 20 weeks. Following a specific phytotherapeutic sheme, patients also took ibuprofen; among aims of the study, there was to know the percentage of patients responding to therapy: 71% of patient taking *H. procumbens* responded to therapy compared to 41% of those on placebo. The difference was statistically significant (p=0.041) (method of statistical analysis not indicated).

In 2002, Biller, treated 78 osteoarthritis patients with knee pain with 4500 mg/day of hydroalcoholic extract of *H. procumbens* (60% alcohol; less than 30 mg/day harpagoside) or placebo for 20 weeks. Patients also took 800 mg/day ibuprofen from week 1 to week 8, and 400 mg/day from week 9 to week 16. From week 17 to week 20 they took Grapple plant alone or placebo. The end-points of the study were the percentage of patients responding to therapy, variations in the WOMAC index of pain and non-recourse to ibuprofen in weeks 17-20. Ninety percent of patients in the grapple group responded to therapy compared to 80% in the placebo group. Mean use of ibuprofen in weeks 17-20 was one tablet/day in the *H. procumbens* group and 5 tablets in the placebo group. The plant was therefore more effective than placebo.

In 2007, Chrubasik *et al*., recruited 114 patients (56 with chronic nonspecific lower back pain, 37 with osteoarthritic knee and 21 with osteoarthritic hip pain) into a survey of the effects of Doloteffins at a dose providing 60 mg harpagoside per day for up to 54 weeks. Their symptoms and well-being were monitored at 4-6 week intervals by disease-specific and generic outcome measures, and patients also kept a diary of their pain and need for rescue medication. The principal analyses were based on Intention to Treat (ITT) with Last Value Carried Forward (LOCF). Multivariate Analysis of Variance (MANOVA) indicated appreciable overall improvement during the survey,

similar in the back, knee and hip groups. In separate ANOVAs, most of individual outcome scores decreased significantly over time. Multiple regression analysis indicated that changes from baseline were independent of patient characteristics. Additional analgesic requirements (which were very modest) declined during the year of the survey. "Response during treatment" was achieved in 75% of patients, and was reflected in the percentages of those who rated the treatment "good" or "very good". Side-effects were few and minor.

In 2007 Warnock and coworkers, published a 8-week open label clinical trial to examine the effectiveness of a Devil's claw preparation (Bioforce, Scotland, United Kingdom) in the treatment of arthritis and other rheumatic conditions, such as arthritic dominant hands. A particular form of osteoarthritis is hand osteoarthritis; this form of arthritis does not seem to depend on any evident cause and mainly affects certain joints of the hand, especially the distal interphalangeal joints, which gradually develop hard swellings known as Heberden nodules, the proximal interphalangeal joints, the tumefactions of which are known as Bouchard nodules, and the metacarpal trapezium, which often becomes partly dislocated. The structural changes (reduced thickness of joint cartilage, sclerosis, subchondrial geodes and osteophyte formation) cause chronic pain and dysfunction with relapsing-remitting acute phases related to joint misalignment (distress of compartmental capsule and ligaments) and secondary synovitis.

Radiologically, the disease is characterised by restriction of the joint line, small pseudocysts and marginal osteophytes. Subluxation can occur with time. The disease is not accompanied by changes in humoral parameters. It mainly affects women over 40 years of age and is progressive, though the speed of progression varies from person to person.

No drugs have yet been demonstrated to slow or arrest progression of joint damage, though cartilage protectors such as diacerein and glycosaminoglycans are widely used. NSAIDs are usually used to control pain and have the advantage of also controlling secondary inflammatory reactions. However, NSAIDs are contraindicated in cases of gastritis, gastric ulcer, kidney failure, heart failure and hypertension and cannot be taken with other drugs such as oral anticoagulants. They have frequent gastrointestinal, renal and cardiovascular side-effects.

In the study of Warnock *et al*. (2007) patients aged 18-75 years with mild to moderate rheumatic disorders were enrolled. The patients received a daily dose of 2 Devil's claw tablets containing a total daily dose of 960 mg Devil's claw dry extract made with 60% ethanol (DER 1.5-3:1). Effectiveness was measured using a global assessment of pain function and stiffness in the effected area with a numerical rating scale (NRS), as well as joint-specific assessments. The WOMAC scale was used to assess effects on arthritic dominant hands. In addition, a general assessment of perceived effectiveness on a 6-point scale, onset of action, the SF-12 Quality of Life Questionnaire, and daily patient diaries were used to assess effectiveness.

A total of 222 patients were included in the Intention to Treat (ITT) analysis. Compliance at week 8 was 75-100% for 140 patients. The average global scores for pain, stiffness, and function were significantly improved from baseline to weeks 4 and 8 ($P<0.0001$ for both). The average scores for pain in the hand, wrist, elbow, shoulder, hip, knee and back were significantly improved from baseline to week 8 ($P<0.05$) and week 4 ($P<0.01$) (except for right elbow). In addition, the average WOMAC subscale scores (knee: n=114, hip: n=68) were significantly improved from baseline to weeks 4 and 8 ($P<0.0001$ for both). The average Algofunctional Hand Osteoarthritis Index (n=113) scores were also significantly improved from baseline to week 8 ($P<0.0001$). Average finger floor distances of patients with back pain (n=81) were significantly improved from baseline to week 8 ($P<0.0001$).

In addition, more than half of the patients rated the Devil's claw treatment as "excellent" or "good"; and investigators rated the Devil's claw extract's effectiveness as "excellent" or "good" in more than half of the patients. A total of 171 out of 222 patients (77%) reported a beneficial effect due to the devil's claw tablets. Of these patients, 104 reported feeling an effect between 1 and 4 weeks from baseline. Average scores for pain, daily function, and stiffness in the patient's daily diaries were significantly improved from week 1 to weeks 2 and 8 ($P<0.0001$) for both). In addition, the average SF-12 scores on the physical and emotional subscales were significantly improved from baseline to weeks 4 and 8 (n=207, $P<0.0001$ for both). Almost half (44.8%) of patients taking analgesics for rheumatic disorders at baseline had reduced their dosage at week 8; and 26% had completely stopped taking analgesics at week 8. There were no significant changes in blood parameters, liver function, or vital signs during the study. A total of 49 possibly or probably drug-related adverse events were reported, none considered serious. The majority of the adverse events reported were mild to moderate gastrointestinal complaints. The tolerability of the devil's claw tablets was rated as "good" by the majority (87.4%) of patients. In addition, 74.3% of the patients stated that they would take the devil's claw tablets again.

Effect on smooth muscle contraction. A number of studies have shown that dry extract of Devil's claw, up to 40 µg/ml, slightly increases the amplitude and tone of contractions of isolated jejunum. At higher doses tone decreases and the

amplitude of contractions declines sharply. The same biphasic effect on contractions has also been observed in isolated rabbit uterus (Occhiuto *et al*., 1985).

***Effect on cardiovascular system*.** Intragastric administration of 100 mg/kg body weight of an aqueous or methanol extract of *H. procumbens* root protected rats against ventricular arrhythmias induced by epinephrine-chloroform and calcium chloride (Circosta *et al*., 1984). Intraperitoneal administration of 25 mg/kg bw of a methanol extract of *H. procumbens* roots inhibited cardiac arrhythmias induced by aconitine, epinephrine-chloroform and calcium chloride in fasted rats. Intragastric administration of 300-400 mg/kg bw of a methanol extract of *H. procumbens* root to normotensive rats reduced heart rate and arterial blood pressure (Circosta *et al*., 1984). Another studies have demonstrated that lower doses of the extract have slight negative chronotropic and positive inotropic effects (Occhiuto *et al*., 1985), whereas larger doses have a marked inotropic effect, with reduction in coronary blood flow. The inotropic effect is attributed to harpagide (Costa *et al*., 1985).

***Antidyspeptic activity*.** As bitter tonic *H. procumbens* has similar effects to gentian with prokinetic effects on gastrointestinal smooth muscle and is indicated in cases of hyposecretory dyspepsia with loss of appetite (WHO, 2007).

A decoction of *Radix Harpagophyti* is one of the strongest bitter tonics known (Weiss and Fintelmann, 2000). Ingestion of an infusion prepared from the root (dose not specified) over a period of several days led to an improvement in the symptoms of disorders of the upper part of the small intestine, which were accompanied by disturbances of choleresis and bile kinesis (Weiss and Fintelmann, 2000). It has been proposed that, because the root is very bitter, is a good stomachic and stimulates the appetite, it may also be useful for the treatment of dyspeptic complaints (Jaspersen-Schib, 1989; Czygan and Krüger, 1977).

Pharmacokinetics. Commercial extract of *H. procumbens* taken orally has been shown to produce a plasma peak of harpagoside in humans within 1.3-2.5 h (Loew *et al*., 2001). Iridoid glycosides are metabolised by human intestinal flora to aucubin B, a pyridine alkaloid. The reaction seems to be catalyzed by the enzyme α-glycosidase in the presence of ammonium ions (Baghdikian *et al*., 1999a, 1999b).

A special role is certainly played by gastric acid which may activate or inactivate iridoid glycosides. It has been suggested that the products of acid hydrolysis have antiinflammatory and antirheumatic activity and that contact with gastric secretions is important. Others sustain that glycosides are more active than the corresponding genines and that gastric acid disactivates the active principle. Indeed, aqueous extract reduces carrageenan-induced edema in rats if administered intraperitoneally or intraduodenally, but not when taken orally (Soulimani *et al*., 1994).

Toxicity. Long-term toxicity does not seem to have been studied. Certain authors underline the need for such studies, particularly in view of the widespread use of the plant in phytotherapy and as complementary therapy, especially in cases in which prescription of anti-inflammatory drugs is unjustified, at least in the early stages of disease. Pharmacological studies with experimental animals have shown that *H. procumbens* is well tolerated: the i.p. DL_{50} of harpagoside and harpagide in mice is 1 and 3.2 g/kg, respectively (Cardini, 2006).

Side-effects. Mild infrequent gastrointestinal symptoms were reported in clinical trials (Chrubasik *et a*l., 1999; Belaiche, 1982) and in cases of known hypersensitivity. Digestive side-effects include diarrhoea (Chantre *et al*., 2000), dyspepsia and sense of satiety (Wegener and Lupke, 2003), though the risk of side-effects is much less than that with synthetic analgesics (Chrubasik, 2004) and the plant is very well tolerated (Chrubasik, 2004).

Contraindications. *Radix Harpagophyti* is contraindicated in cases of gastric and duodenal ulcer and known hypersensitivity to the roots, because the bitter taste stimulates peptic acid (Blumenthal, 1998; Hänsel *et al*., 1993). Owing to a lack of safety data, *Radix Harpagophyti* should not be used during pregnancy and nursing, due to possible oxytocic effects (Mabey *et al*., 1988).

Since *H. procumbens* has antiarrhythmic activity (Circosta *et al*., 1984), it should not be taken in association with antiarrhythmic drugs. A possible interaction with anticoagulants such as Warfarin, that could promote bleeding, has been reported (Heck *et al*., 2000; Shaw *et al*., 1997). It is well known that a number of herbs and foods alter the metabolism of Warfarin by acting on cytochrome P450. Budzinski *et al*. (2000) demonstrated that Devil's claw did not inhibit the activity of cytochrome P450 isoenzymes *in vitro*. On the other hand, Unger and Frank (2004) described a liquid chromatography/mass spectrometry method with automated online extraction to simultaneously determine the *in vitro* inhibitory potency of herbal extracts on six major human drug-metabolising cytochrome P450 enzymes. The authors concluded that popular herbal remedies, including Devil's claw root, could be identified as inhibitors of the applied CYP enzymes with IC(50) values between 20 and 1000 µg/ml. Unger and Frank (2004)

showed that this inhibition could be the reason for drug interactions of clinical significance.

These controversial results are not easy to compare; more studies are needed. However, on the basis of pharmacological considerations, ESCOP (2003) does not exclude interaction with antiarrhythmics and warns against possible interaction with Warfarin.

Other precautions. No information is available on precautions concerning drug and laboratory test interactions, carcinogenesis, mutagenesis, impairment of fertility, teratogenic effects during pregnancy or paediatric use.

Preparations. Dried root for decoctions and infusions, powdered root or extract in capsules, tablets, tinctures and ointments (Bisset, 1994; Iwu, 1993).

Store in the dark in a sealed container.

Posology. (Unless otherwise indicated). Daily dose for loss of appetite: 1.5 g of root in decoction, 3 ml of tincture (1:10, 25% ethanol); for painful arthritis or tendonitis 1.5-3.0 g of root in decoction, three times a day, 1-3 g of root or equivalent (WHO, 2007).

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