ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Isolation and Characterization of *Polygonum equisetiforme* Flavonoids and Their Acaricidal Activity against *Tetranychus urticae* Koch.

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ABSTRACT

Acaricidal activity assessment against *Tetranychus urticae* Koch (Acari: Tetranychidae) of different extracts of *Polygonum equisetiforme* (Polygonaceae) aerial parts revealed that butanol extract has the highest activity. Processing of this fraction using repeated column and thin layer chromatographic techniques resulted in the isolation of five flavonoids, which have identified NMR as; quercetin (1), avicularin (2), isoquercitrin(3), betmidin (4) and myricitrin (5). All the five isolated flavonoids showed acaricidal activity using leaf-dip technique against *T. urticae* Koch larvae in the following decreasing activity order; quercetin (LC_{50} =11.28 ppm), avicularin (46.72 ppm), isoquercitrin (63.77 ppm), myricitrin (169.66 ppm) and betmidin (188.11 ppm). While the activity against the adult females was found to be quercetin (LC_{50} =16.28 ppm), isoquercitrin (93.38 ppm), avicularin (98.57 ppm), betmidin (161.00 ppm) and myricitrin (206.68 ppm) after 7 days of treatment. This is the first report on isolation of the two flavonoids betmidin (4) and myricitrin (5) from *P. equisetiforme.*

Keywords: Acaricidal activity, *Tetranychus urticae* Koch, *polygonum equisetiforme*, polygonaceae, Flavonoids, quercetin, isoquercitrin, avicularin, betmidin, myricitrin.

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ISSN: 0975-8585

INTRODUCTION

Tetranychus urticae Koch is a worldwide pest, feeding on a large variety of plant families in Egypt, attacking cotton, fruit trees and vegetables. This mite showed readily and rapidly resistance to synthetic acaricides, moreover, synthetic acaricides comprises high toxic impact towards mammals and birds and unfriendly to environment. Therefore, it is recommended to search for natural acaricides from plants, which has stimulated us to examine some available Egyptian plants for these desired botanicals [1-3].

Polygonum equisetiforme (Polygonaceae) was used as a cure lotion for treating poisonous snakebites in ancient Egypt. [4] In addition, it has anti-inflammatory, astringent therapeutically effects [5] and its roots water extract was applied for treating kidney and urinary tract diseases [6].

Ethanol extracts from *P. equisetiforme* exhibited strong antimicrobial activity against Gram-positive bacteria [7, 8]. Polysaccharides were isolated from roots of *P. equisetiforme* found to be effective natural antioxidants, with little antitumor activity. [9] Previous phytochemical investigation of *Polygonum equisetiforme* (Polygonaceae) revealed the isolation of quercetin, quercetin-3-O-rhamnoside, quercetin-3-O-glucuronide, quercetin-3-O-arabinoside and isorhamnetin [7]. In addition the isolation and identification of apigenin-6-C-arabino-pyranosyl-8-C-glucopyranoside, apigenin-6,8 di C-gluco-pyranoside, quercetin-3-O-gluco-pyranoside, quercetin-3,7-di-O-gluco-pyranoside, rhamnetin-3-O-rhamno-pyranoside, myricetin (3,5,7,3',4',5'-hexahyroxyflavone) and gallic acid were reported [10]. We report here the flavonoids isolated from *Polygonum equisetiforme* as natural acaricides against *T. urticae* Koch.

MATERIALS AND METHODS

General

NMR experiments were performed on a Bruker AMX 400 instrument standard pulse sequences operating at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR. Chemical shifts are given in δ (ppm) relative to TMS as internal standard material and the coupling constants (*J*) are in Hz. UV spectra were recorded on Shimadzu, model 2401.

Chemicals

Thin layer chromatography and preparative TLC were performed on silica gel (Kieselgel 60, F 254) of 0.25 mm thickness. Pet. ether (60-80°C), methylene chloride, ethyl acetate and methanol were obtained from Adwic Company.

Plant material

The aerial parts of *Polygonum equisetiforme* was collected from Al-Aresh city, Sinai at April, 2009, identified by Prof. Dr. Loutfy Boulos, Professor of Botany, Faculty of Science, Alexandria University, Egypt.

ISSN: 0975-8585

Extraction and isolation

The dried powdered aerial parts of the plant (1.15 kg) were extracted by a soxhlet apparatus using pet. ether, methylene chloride, ethyl acetate and methanol, successively. The solvents were evaporated under vacuum to give pet. ether fraction (7.79 g), methylene chloride fraction (5.78 g), ethyl acetate fraction (6.32 g) and methanol fraction (116.75 g), which was further washed by butanol to give the butanol fraction (4.35 g). Butanol fraction (4.35 g) was subjected to Sephadex LH-20 CC using MeOH as solvent to give ten subfractions. The third subfraction has been applied on TLC Silica gel plates using EtOAc–MeOH–H_2O (98:1:1) as a developing system to afford compound (3), (26 mg, R_f 0.13). The fourth subfraction was purified on silica gel TLC, developed by the same previous system to afford compound (2), (42 mg, R_f 0.54), (4), (12 mg, R_f 0.33) and (5), (14 mg, R_f 0.20). The fifth subfraction has been separated on TLC Silica gel plates using EtOAc to give compound (1) (59 mg, R_f 0.85).

Quercetin 3-*O*-α-L-arabinofuranoside (Avicularin) (2). Yellow needle crystals. R_f 0.54; UV (MeOH, max) 256, 358 nm; ^{1}H NMR (400 MHz, CD_3OD, δ , ppm, J, Hz): 7.54 (br s, H-2'), 7.51 (br d, J = 8.3, H-6'), 6.92 (br d, J = 8.3, H-5'), 6.41 (br s, H-8), 6.23 (br s, H-6), 5.48 (br s, H-1''), 4.35 (br d, J = 1.3, H-2''), 3.95 (dd, J = 3.8, 8.3, H-4''), 3.93 (m, H-3''), 3.55 (m, 2H-5''); ^{13}C NMR (100 MHz, δ, ppm): 179.9 (C-4), 165.9 (C-7), 163.0 (C-5), 159.3 (C-2), 158.5 (C-9), 149.8 (C-4'), 146.3 (C-3'), 134.9 (C-3), 123.0 (C-1'), 122.9 (C-6'), 116.4 (C-2'), 116.8 (C-5'), 109.5 (C-1''), 105.6 (C-10), 99.8 (C-6), 94.7 (C-8), 88.0 (C-4''), 83.2 (C-2''), 78.6 (C-3''), 62.5 (C-5'').

Quercetin 3-*O* -β-D-glucopyranoside (Isoquercitrin) (3). Yellow powder. R_f 0.13; UV (MeOH) 258, 360 nm; ^{1}H NMR (400 MHz, CD_3OD, δ , ppm, J, Hz): 7.86 (d, J = 1.7, H-2'), 7.59 (dd, J = 1.7, 8.4, H-6'), 6.87 (d, J = 8.4, H-5'), 6.36 (br s, H-8), 6.18 (br s, H-6), 5.12 (d, J = 7.8, H-1''), 3.65 (dd, J = 5.1,11.1, H-6''), 3.57 (dd, J = 5.1,11.1, H-6''), 3.83 (m, H-2''), 3.58 (m, H 3''), 3.87 (m, H-4''), 3.51 (m, H-5''); ^{13}C NMR (100 MHz, δ , ppm): 179.2 (C-4), 168.3 (C-7), 162.8 (C-5), 158.6 (C-2), 158.5(C-9), 149.9 (C-4'), 145.8 (C-3'), 135.7 (C-3), 122.9 (C-1'), 122.8 (C-6'), 117.7 (C-5'), 116.1 (C-2'), 105.6 (C-10), 104.9 (C-1''), 100.6 (C-6), 95.2 (C-8), 77.1 (C-5''), 75.1 (C-3''), 73.2 (C-2''), 70.0 (C-4''), 61.9 (C-6'').

Myricetin 3-*O*-α-L-arabinofuranoside (Betmidin) (4). Yellow powder. R_f 0.33; UV (MeOH) 256, 354 nm; ^{1}H NMR (400 MHz, CD_3OD, δ , ppm, J, Hz): 7.15 (br s, H-2'), 7.15 (br s, H-6'), 6.40 (d, J = 2, H-8), 6.22 (d, J = 2, H-6), 5.48 (br s, H-1''), 4.21 (br d, J = 1.8, H-2''), 3.95 (m, H-3''), 3.95 (m, H-4''), 3.51 (t, J = 3.4, 2H-5''); ^{13}C NMR (100 MHz, δ, ppm): 179.9 (C-4), 166. 0 (C-7), 163.0 (C-5), 159.3 (C-2), 158.5 (C-9), 146.8 (C-3'), 146.8(C-5'), 137.9 (C-4'), 134.8 (C-3), 121.9 (C-1'), 109.4 (C-6'), 109.4 (C-2'), 109.4 (C-1''), 105.4 (C-10), 99.9 (C-6), 94.8 (C-8), 88.0 (C-4''), 83.2 (C-2''), 78.7 (C-3''), 62.5 (C-5'').

Myricetin 3-O-α-L-rhamnopyranoside (Myricitrin) (5). Yellow powder. R_f 0.20; UV (MeOH) 256, 350 nm; ^{1}H NMR (400 MHz, CD_3OD, δ , ppm, J, Hz): 6.97 (br s, H-2'), 6.97 (br s, H-6'), 6.37 (d, J = 2, H-8), 6.21 (d, J = 2, H-6), 5.33 (d, J = 1.1, H-1''), 4.21 (m, H-2''), 3.81 (dd, J = 3.1, 9.4, H-3''), 3.51 (dq, J = 6.1, 9.4, H-5''), 3.33 (t, J = 9.4, H-4''), 0.98 (d, J = 6.2, H-6''); ^{13}C NMR (100 MHz, δ, ppm): 179.6 (C-4), 166. 0 (C-7), 163.1 (C-5), 159.3 (C-2), 158.5 (C-9), 146.8 (C-3'), 146.8(C-5'),

137.8 (C-4'), 136.2 (C-3), 121.9 (C-1'), 109.5 (C-6'), 109.5 (C-2'), 105.7 (C-10), 103.6 (C-1''), 99.8 (C-6), 94.7 (C-8), 73.3 (C-4''), 72.0 (C-3''), 72.0 (C-5''), 71.8 (C-2''), 17.6 (C-6'').

Maintance of spider mite colony

Colony of spider mite *Tetranychus urticae* Koch was reared under laboratory condition (25±2 °C and 60±5 % R.H) at Plant Protection Research Institute branch, Dakahlia Governorate. This study colony was isolated from heavily infested castor oil plant leaves. Spider mite colony was reared on castor oil leaves. These leaves were cleaned and placed on moisten cotton wool pad in Petri dishes. This colony was left for one year under the precious conditions in order to get a homogenous and sensitive colony. Spider mite individuals were transferred to the leaves by aid of fine camels hair brush. Breeding leaves were changed twice weekly at summer and once weekly at winter. Adding water was done twice daily to prevent escaping of *T. urticae* individuals.

Assessment of acaricidal activity

In this respect, laboratory experiments are conducted to evaluate the activity of various tested plant extracts against *T. urticae* mobile stages (larvae and adult females). The leaf-dip technique was used [11].

The indication of mortality was chosen as the failure of mites to respond positively by leg movement followed light brooding with a fine brush. Mortality percentages were determined and corrected by using Abotts (1925) formula and they are statistically analyzed to estimate LC_{50}, LC_{90} and slope values according to Finney (1971) [12, 13]. Toxicity index was computed for different extracts and the isolated compounds by comparing these materials with the most effective extracts or isolated compounds using Sun's (1950) equation [14].

$$\text{Toxicity index} = \frac{LC_{50} \text{ of compound A}}{LC_{50} \text{ of compound B}}$$

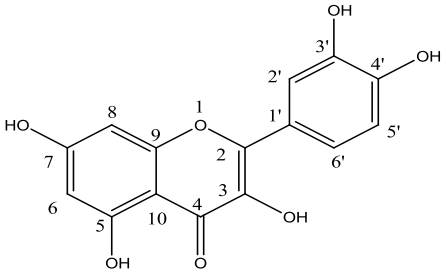
Where:
A is the most effective compound
B is the tested compound

RESULTS AND DISCUSSION

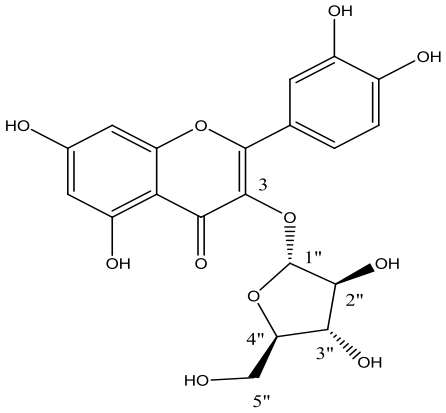
Processing of the plant aerial parts has resulted in four fractions; pet. ether, methylene chloride, ethyl acetate and butanol fractions (*cf*. Experimental). The acaricidal activity indicated that butanol fraction was the most effective fraction against the larvae and adult females of *T. urticae* after 7-days of treatment that guided and stimulated us to search for its bioactive components. Repeated column and thin layer chromatography has resulted in the isolation of five flavonoids. They were characterized by NMR spectroscopy (*cf*. Experimental).

ISSN: 0975-8585

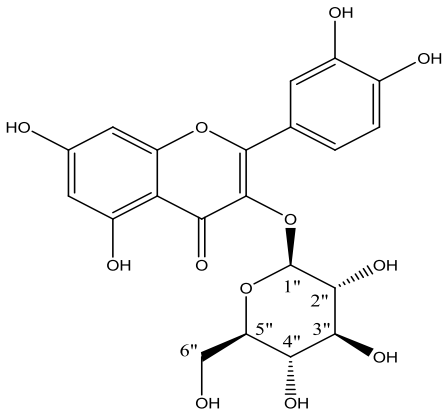
Examination of NMR spectra of the five isolated compounds from the butanol fraction in addition to their UV absorption spectra indicated that they belong to flavonoids. The ^{1}H-NMR data of compound (1) indicated that ring A is 5,7-disubstituted, as shown by two meta-located protons at δ 6.15 ppm (1H, br s, H-6) and δ 6.39 ppm (1H, br s, H-8). On the other hand, the observation of ABX system at δ 7.63 ppm (1H, br s, H-2'), δ 6.85 ppm (1H, br d, J=8.4 Hz, H-5') and δ 7.50 ppm (1H, dd, J=2.3, 8.4 Hz, H-6') has suggested a 3',4'-disubstituted ring B, which was identified as quercetin [11].



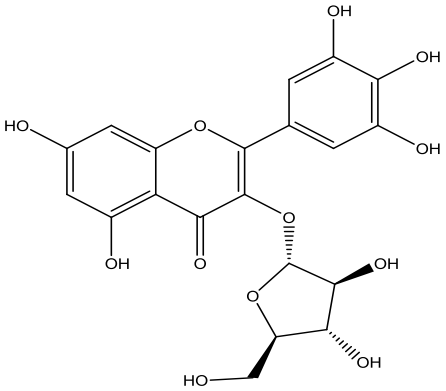
Quercetin (1)



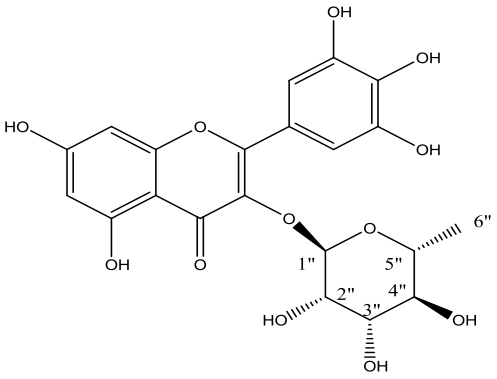
Avicularin (2)



Isoquercitrin (3)



Betmidin (4)



Myricitrin (5)

ISSN: 0975-8585

A 3-O-substituted quercetin structures were indicated for compounds (2) and (3) due to the corresponding anomeric protons at δ 5.48 ppm (1H, br s) and δ 5.12 ppm (1H, d, J=7.8 Hz), characteristic for arabinofuranosyl and glucopyranosyl moieties, respectively (Table 1). The structure of compounds (2) and (3) were characterized as quercetin 3-O-α-L-arabinofuranoside (avicularin) and quercetin 3-O-β-D-glucopyranoside (isoquercitrin) by H-H COSY, HSQC and HMBC and comparison with literature data [15].

Table 1: NMR data (δ ppm) for compounds 2-5.

Position	(2)		(3)		(4)		(5)	
	1H (multiplicity, J)	^{13}C	1H (multiplicity, J)	^{13}C	1H (multiplicity, J)	^{13}C	1H (multiplicity, J)	^{13}C
2	-	159.31	-	158.59	-	159.35	-	159.39
3	-	134.9	-	135.71	-	134.86	-	136.27
4	-	179.97	-	179.22	-	179.99	-	179.62
5	-	163.03	-	162.84	-	163.03	-	163.17
6	6.23 (1H, brs)	99.87	6.18 (1H, brs)	100.65	6.22 (1H, d, J=2 Hz)	99.99	6.21 (1H, d, J=2 Hz)	99.87
7	-	165.99	-	168.33	-	166.40	-	166.08
8	6.41(1H, brs)	94.76	6.36(1H, brs)	95.25	6.40(1H, d, J=2 Hz)	94.82	6.37(1H, d, J=2 Hz)	94.73
9	-	158.53	-	158.49	-	158.58	-	158.50
10	-	105.61	-	105.64	-	105.46	-	105.79
1'	-	123.08	-	122.90	-	121.99	-	121.91
2'	7.54 (1H, brs)	116.43	7.86 (1H, d, J=1.7 Hz)	116.07	7.15 (1H, s)	109.41	6.97 (1H, s)	109.57
3'	-	146.32	-	145.82	-	146.85	-	146.83
4'	-	149.82	-	149.97	-	137.98	-	137.88
5'	6.92 (1H, brd, J=8.3 Hz)	116.83	6.87 (1H, brd, J=8.4 Hz)	117.71	-	146.85	-	146.83
6'	7.51 (1H, brd, J=8.3 Hz)	122.96	7.59 (1H, dd, J=1.7, 8.4 Hz)	122.88	7.15 (1H, s)	109.41	6.97 (1H, s)	109.57
1''	5.48 (1H, brs)	109.53	5.12 (1H, d, J=7.8 Hz)	104.96	5.48 (1H, brs)	109.41	5.33 (1H, d, J=1.1 Hz,	103.60
2''	4.35 (1H, brd, J=1.3 Hz)	83.27	3.83 (1H, m)	73.18	4.21 (1H, brd, J=1.8 Hz)	83.24	4.21 (1H, m)	71.87
3''	3.93 (1H, m)	78.68	3.58 (1H, m)	75.13	3.95 (1H, m)	78.76	3.81 (1H, dd, J=9.4, 3.1 Hz)	72.11
4''	3.95 (1H, dd, J=8.3, 3.8 Hz)	88.02	3.87 (1H, m)	70.01	3.95 (1H, m)	88.05	3.33 (1H, t, J=9.5 Hz)	73.34
5''	3.55 (2H, m)	62.53	3.51 (1H, m)	77.14	3.51 (2H, t, J=3.4 Hz)	62.56	3.51 (1H, dq, J=9.4, 6.1 Hz)	72.02
6''a	-	-	3.65 (1H, dd, J=5.1, 11.1 Hz)	61.92	-	-	0.98 (3H, d, J=6.2 Hz)	17.66
6''b			3.57 (1H, dd, J=5.1, 11.1 Hz)					

Compounds (4) and (5) were found to belong to myricetin glycosides, as established by the typical UV absorption (λ_{max} 256, 350 nm) and NMR spectra (Table 1). The sugar residues were determined by the characteristic sugar anomeric proton signals at δ 5.48 ppm (1H, br s) and δ 5.33 ppm (1H, d, J=1.1 Hz), which were assigned to arabinofuranosyl, and rhamnosyl moieties, respectively. Therefore, compounds (4) and (5) were identified as myricetin 3-O-

α-L-arabinofuranoside (betmidin) and myricetin 3-O-α-L-rhamnopyranoside (myricitrin), respectively. They were confirmed by H-H COSY, HSQC and HMBC and comparison with literature data [16].

It is worthwhile mentioning here, that this is the first report of betmidin (4) and myricitrin (5) from *P. equisetiforme* (Polygonaceae).

Toxicity effect of the plant fractions to larvae and adult females of *Tetranychus urticae* (Koch) after 7-days of treatment

The susceptibility of the larvae and adult females of *T. urticae* to various solvent plant extract fractions after 7-days of treatments is reported in table (2). The descending order of the acaricidal activity of both larvae and adult females at LC_{50} and LC_{90} levels were butanol fraction, ethyl acetate fraction, methylene chloride fraction and pet. ether fraction. The slopes of the toxicity lines were calculated to be fluctuated and increased from 0.919 in the ethyl acetate fraction to 1.625 in the pet. ether fraction in case of larvae and 1.161 in ethyl acetate fraction to 1.858 in the pet. ether fraction for the adult females. The other fractions lines came between these two fraction lines.

The toxicity index of the LC_{50} values, as shown from table (2), indicated that butanol fraction was the most effective plant fraction against larvae and adult females of *T. urticae* after 7-days of treatment, followed by ethyl acetate fraction, methylene chloride fraction while pet. ether fraction was the least effective one.

Toxicity effect of the isolated flavonoids to larvae and adult females of *Tetranychus urticae* (Koch) after 7-days of treatment

Table (3) showed the susceptibility of both larvae and adult females of *T. urticae* to the isolated flavonoids (1-5). Data revealed that quercetin (1) exhibited the highest efficiency against both larvae and adult females after 7- days of treatment and this was proved from the toxicity index of the isolated flavonoids.

It is most likely from the examination of the chemical structure of the isolated flavonoids and their toxicity index at LC_{50} that substitution of the hydrogen atom at position 3' in ring B decreases the acaricidal activity.

ISSN: 0975-8585

Table 2: Toxicity of plant fractions against larvae and adult females of *T. urticae* after 7- days of treatment.

Plant extract	Larvae				Adult females			
	LC_{50} (ppm) and confidence limits at 95%	LC_{90} (ppm) and confidence limits at 95%	Slope	Toxicity index at LC_{50} value	LC_{50} (ppm) and confidence limits at 95%	LC_{90} (ppm) and confidence limits at 95%	Slope	Toxicity index at LC_{50} value
Pet. ether fraction	101.60 20.49 172.89	624.85 412.21 1718.29	1.625±0.464	8.63	299.76 128.69 440.50	1467.90 999.94 3405.00	1.858±0.449	6.30
Methylene Chloride fraction	98.72 17.78 170.10	894.81 516.63 5101.58	1.339±0.403	8.88	129.50 24.76 218.51	1575.56 763.87 25017.60	1.181±0.376	14.58
Ethyl acetate fraction	23.73 6.37 44.60	588.61 294.20 2677.46	0.919±0.204	36.93	41.57 18.17 65.01	527.59 269.22 2665.92	1.161±0.270	45.43
Butanol fraction	8.76 3.68 15.49	170.01 87.41 533.91	0.995±0.173	100.00	18.89 3.05 32.87	233.16 98.47 906.94	1.349±0.408	100.00

Table 3: Toxicity of isolated compounds against larvae and adult females of *T. urticae* after 7- days of treatment.

Isolated Compounds	Larvae				Adult females			
	LC_{50} (ppm) and confidence limits at 95%	LC_{90} (ppm) and confidence limits at 95%	Slope	Toxicity index at LC_{50} value	LC_{50} (ppm) and confidence limits at 95%	LC_{90} (ppm) and confidence limits at 95%	Slope	Toxicity index at LC_{50} value
Quercetin (1)	11.28 2.84 18.46	93.30 54.47 459.93	1.397±0.400	100.00	16.28 7.29 22.95	81.07 46.83 663.37	1.838±0.582	100.00
Avicularin (2)	46.72 10.66 90.45	957.66 475.70 4851.52	0.977±0.236	24.15	98.57 15.10 173.11	995.50 552.62 7443.61	1.276±0.397	16.51
Isoquercitrin (3)	63.7688 11.31 110.10	764.60 372.79 11456.51	1.188±0.378	17.69	93.38 33.46 153.08	1478.92 704.75 10451.69	1.068±0.267	17.43
Betmidin (4)	188.11 83.21 281.29	1580.04 832.70 10278.24	1.387±0.374	6.00	161.00 98.88 231.93	1418.64 775.49 5026.86	1.356±0.270	10.11
Myricitrin (5)	169.66 109.90 239.17	1291.56 740.44 3908.51	1.454±0.274	6.65	206.68 126.61 314.64	2432.75 1124.17 14791.31	1.197±0.261	7.88

ISSN: 0975-8585

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