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Biochemical composition of two giant pill-millipedes of the Western Ghats of India

CHEVIRI N. AMBARISH[1] and KANDIKERE R. SRIDHAR[2]

[1] Department of Biochemistry, St. Aloysius College, Mangalore 575 003, Karnataka, India
[2] Department of Biosciences, Mangalore University, Mangalagangotri, Mangalore 574 199, Karnataka, India

Corresponding author: Kandikere R. Sridhar, email: kandikere@gmail.com

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Abstract: Many invertebrates have an unexpected nutraceutical potential and are of nutritional or ethnomedicinal significance to many tribals throughout the world. The giant pill-millipedes of the genus *Arthrosphaera* are traditionally used as natural medicines by tribals in the Western Ghats of India. In this study, two species of pill-millipedes (*Arthrosphaera fumosa* and *A. magna*) were subjected to proximate and biochemical analysis to ascertain their nutritional potential. Bodies of *A. fumosa* and *A. magna* (after removal of their intestines) had a low protein content (8-15%) and high quantity of carbohydrates (40-41%). They were rich in many essential elements, especially in calcium. The essential amino acids of pill-millipedes were in high quantities. The level of glycine was the highest, followed by lysine and serine. The fatty acid methyl esters (FAMEs) of males and females consist of high quantities of unsaturated fatty acids. The mono-unsaturated fatty acids were more abundant than poly-unsaturated fatty acids. Palmitic and oleic acids were dominant saturated and unsaturated fatty acids, respectively. The study has revealed for the first time that pill-millipedes of the Western Ghats of India constitute a good source of essential minerals, essential fatty acids, and essential amino acids. Being valuable contributors of organic manure by processing recalcitrant plant lignocellulosic wastes, pill-millipedes become part and parcel of organic farming as well as future nutraceutical sources.

Keywords: *Arthrosphaera*, millipedes, proximate composition, minerals, amino acids, fatty acids

INTRODUCTION

Invertebrates are megadiverse and they have been considered as one of the potential future nutritional resources for the teeming human population (Rumpold & Schluter 2013). Many invertebrates possess unexpected nutraceutical properties and are used as food by the local or ethnic population (Enghoff et al. 2014). Consumption of insects as food was considered as one of worldwide practices and still serves as traditional or ethnic food in many countries, particularly those facing short supply of food and threats of food scarcity (Tommaseo-Ponzetta 2005; Christensen et al.

2006). On the other hand, many countries use food products of insect origin rather than consuming the whole insects (Perlės 2006). Other than insects, however, the use of arthropods as sources of food is very much limited to the tribes of Africa and South East Asia (e.g. China, Korea and Thailand). The body of pill-millipedes consists primarily of a series of tergites, with low muscle mass, and an intestine with unique microbiota (Anderson & Bignell 1980; Wesener & Sierwald 2005). According to Tommaseo-Ponzetta (2005), the low muscle mass in pill-millipedes means they are poor sources of protein, compared to other arthropods. Tergites are rich in calcium and earlier studies showed that their calcium content is up to 13-17% of the dry mass (Reichle et al. 1969; Nakamura & Taira 2005).

Millipedes are the most conspicuous soil animals, but so far they have not been considered as sources of food. Moreover, many millipedes of the orders Glomerida (temperate pill-millipedes), Callipodida, Julida, Polydesmida, Siphonophorida, Spirobolida, and Spirostreptida (Afro-Madagascan millipedes) produce chemical defensive secretions to deter predators (Oonincx et al. 2010). Earlier reports by Hopkin and Read (1992) also state that cylindrical millipedes (superorder: Juliformia) mainly secrete benzoquinones, while flat-backed millipedes (superorder: Merocheta) contain mandelonitrile (derivative of hydrogen cyanide). Later, Enghoff et al. (2014) indicated that the low, sub-lethal dose of cyanide in millipedes helps to activate the innate immune response against malarial parasites. The centipedes were used as many traditional medicines as well as food in South-East Asia (Pemberton 2005). Biochemical profiling of millipedes has revealed their unique nutritional potential, especially from the African continent (Enghoff et al. 2014). Enghoff et al. (2014) have also indicated that the Bobo population of Burkina Faso were traditionally entomophagic and consumed different millipedes as food sources. Unlike temperate pill-millipedes (*Glomeris*), the genus *Arthrosphaera* (order Sphaerotheriida) are devoid of offensive odour or secretions (Wesener et al. 2010) and no detailed study on its significance as an alternative nutritional source is available to date. Pill-millipedes in the tropics are the pre-Jurassic elements and their complete profile of minerals, amino acids, and fatty acids are still unknown, especially from the Indian subcontinent. Thus, the major aim of this paper is to evaluate the biochemical profile of males and females of two common pill-millipede species, endemic to the forests and plantations of the Western Ghats of India.

MATERIAL AND METHODS

Samples

Adult male and female individuals of pill-millipedes *Arthrosphaera fumosa* Pocock and *A. magna* Attems, collected from the Western Ghat forests and plantations, were sacrificed and their intestines were removed. Later, their bodies were washed in sterile distilled water, lyophilized, and powdered before biochemical analysis.

Proximate composition

Moisture content of lyophilized sample was determined gravimetrically, on the basis of body weight (b.w.):

moisture (%) = [(fresh b.w. – lyophilized b.w.) ÷ (fresh b.w.)] × 100

Crude protein, total lipids, crude fibre, ash, carbohydrates, and calorific value of the male and female individuals of *Arthrosphaera* spp. were measured based on standard methods. Crude protein (N × 6.25) was assessed by the micro-Kjeldahl method (AOAC 2006), total lipids were extracted by the method of Bligh and Dyer (1959), crude fibre and ash contents were determined gravimetrically according to Aoac (2006) methods, and carbohydrates were estimated by the phenol sulphuric acid method (Dubois et al. 1956). Briefly, carbohydrate solutions (2 ml) were mixed with an aqueous solution of phenol (5%, 1 ml) in a test tube and 5 ml of concentrated sulphuric acid was added and mixed rapidly, incubated for 10 min, vortexed and kept for 20 min in a water bath at room temperature for colour development. The colour complex developed was read at 490 nm by a spectrophotometer (UV-VIS Spectrophotometer-118, SYSTRONICS, Ahmedabad, Gujarat, India). The calorific value (kJ/100 g) was calculated according to the procedure of Ekanayake et al. (1999):
calorific value = (crude protein × 16.7) + (total lipids × 37.7) + (carbohydrate × 16.7)

Mineral analysis

Mineral content (Al, Ca, Cu, Fe, Pb, Mg, Mn, K, Se, Na, Zn, and P) of millipedes was determined according to the procedure of Ramamurthy and Kannan (2009). The lyophilized samples were subjected to scanning electron microscope-energy dispersive spectrometer (SEM-EDS) analysis. The microphotographs were recorded (SEM JEOL model, JSM 6380LA) with an accelerating voltage of 20 keV, at high vacuum (HV) mode as secondary electron image (SEI). The maximum magnification possible in this equipment was 300000 times, with resolution of X varying from 50-100 for all samples. The semi-quantification of elemental analysis was performed to identify the weight percentage of major and minor elements present in the samples (Joel JSM 6380LA-SEM-EDS). The EDS is a popular method for determination of trace elements in environmental samples. The morphological characters obtained from SEM supported by the EDS microanalysis device help to quantify several elements (C, N, Na, K, Ca, Mg, Al, Mn, Fe, Cr, Co, Ni, Cu, Zn, Se, Pb and Cd) in homogeneous moisture-free samples. The SEM photographs and corresponding EDS spectrum were taken for the samples and percentage contents of elements present in the samples were estimated.

Amino acid analysis

The protocols of Hofmann et al. (2003) were followed for amino acid profiling of millipedes. Known amount of lyophilized powder was hydrolysed (HCl, 6N, 15 ml) for 4 hr (145°C) and oxidized samples were used for sulphur-containing amino acids. Samples were allowed to cool after HCl was removed by a rotoevaporator (Büchi Laboratoriumstechnik AG RE121; Switzerland) with a vacuum pump (MC2C; Vacuubrand GmbH, Germany). The internal standard was added to each sample (*trans*-4-(Aminomethyl)-cyclohexanecarboxylic acid; Sigma Aldrich; purity 97%). The derivatization was carried out by esterification with trifluoroacetylation (Brand et al. 1994). The standard amino acids were weighed in reaction vials and dried (in CH_2Cl_2) under a gentle stream of helium with slow heating in an oil bath

(40-60°C) to remove water traces. Next, 12 ml of fresh acidified isopropanol (acetyl chloride, 3 ml + 2-propanol, 12 ml) was added and the mixture was heated (100°C, 1 h). On cooling, the reagent was removed by a gentle stream of helium (60°C). To remove propanol and water, evaporation with 3 successive aliquots of CH_2Cl_2 was used. The dry residue was tri-fluoroacetylated with 200 ml of trifluoroacetic anhydride for overnight at room temperature. An aliquot of this solution was used without any treatment for gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS/MS). The measurements of GC-C-IRMS/MS were carried out using gas chromatograph (Hewlett-Packard 58590 II; Germany), connected through a split with a combustion interface to the IRMS system (Finnigan MAT with GC-C-II to MAT 252; Germany) for the isotopic determination of nitrogen and through a transfer line with a mass spectrometer (GCQ, Finnigan MAT; Germany) for qualitative and quantitative analysis of amino acids based on standards. The capillary column of GC (50 m × 0.32 mm i. d. × 0.5 µm BPX5, SGE), operating with the carrier gas flow (1.5 ml/min) with the following temperature and pressure: initial 50°C (1 min), increased to 100°C at 10°C/min (10 min), increased to 175°C at 3°C/min (10 min) and increased to 250°C/min (10 min) (head pressure, 13 psi, 90 kPa).

Fatty acid analysis

The total lipids extracted by the Bligh and Dyer's (1959) method were used to determine the fatty acid methyl esters (FAMEs) (Padua-Resurreccion & Benzon 1979). First, 5% HCl (0.2 ml) and acetyl chloride (8.3 ml) were added drop-wise to absolute methanol (100 ml) in an ice-jacket. This mixture (0.2 ml) was added to lipids of millipedes (200 mg) in a screw-cap glass vial (15 ml capacity), mixed, vortexed, incubated (70ºC, 10 h) and cooled to room temperature. It was suspended in 500 µl distilled water, and next l00 µl of HPLC-grade n-hexane was added, vortexed and allowed to separate. On separation of two layers, the top hexane layer was aspirated into air-tight microcentrifuge tubes and stored in a refrigerator (4ºC) for assay of FAMEs. The 100 µl of esterified samples in vials were diluted with 900 µl of HPLC grade n-hexane. An aliquot of 1 µl was injected into a gas chromatograph (GC-2010, Shimadzu, Japan) having an auto injector (AOI) and capillary column (BPX-70: length 60 m; internal diameter 0.32 mm; film thickness 0.25 µm). The capillary column was conditioned (10 h) prior to use. The elutant was detected on flame ionization detector (FID), and the amplified signals were transferred and monitored with GC-Solutions software. Quantification of FAMEs was based on the standard mixture (C_4-C_{24}) (Sigma, USA) run under similar conditions of sample analysis. The results were depicted in comparison with standard fatty acids and fatty acids in the internal library.

Data analysis

The differences in proximate composition, minerals, amino acids, and fatty acids between male and female *A. fumosa* and *A. magna* were assessed by Student's *t*-test (SigmaPlot 2008). The concentration and area of each peak of FAMEs was computed using the GC Post-run analysis software (http://www.umich.edu/~mssgroup/docs/GCinstructions.pdf).

RESULTS

Proximate composition

Dry mass was higher in males of *A. magna* compared to females, but without significant difference (Table 1). The percentage of moisture tended to be higher in *A. fumosa* than in *A. magna*, but no significant difference was observed between males and females. Ash content was higher in males than in females ($P < 0.05$) and the opposite was true for crude fibre in *A. fumosa* ($P < 0.01$) and *A. magna* ($P > 0.01$). Crude protein content was higher in females than in males ($P < 0.05$). Total lipid levels were higher in females than in males of *A. fumosa* and *A. magna*. Total carbohydrate content did not significantly differ between males and females. The calorific value was higher in females of *A. fumosa* and *A. magna* than in males ($P < 0.05$).

Table 1. Proximate composition of males and females of *Arthrosphaera* spp

	A. fumosa		*A. magna*	
	male	female	male	female
Dry mass (%)	41.00±0.20^{a}	41.80±1.50^{a}	44.26±0.40^{a}	42.60±0.74^{a}
Moisture (%)	58.96±0.30^{a}	58.90±0.15^{a}	55.70±0.45^{a}	57.50±0.40^{a}
Ash (%)	41.32±0.26^{a}	34.98±0.35^{b*}	39.07±0.41^{a*}	33.69±0.40^{b}
Crude fibre (%)	6.43±0.37^{a}	8.40±0.78^{b**}	7.10±0.10^{a}	7.60±0.15^{a}
Total protein (%)	8.18±0.89^{a}	13.60±0.5^{b*}	10.06±0.38^{a}	14.50±0.23^{b*}
Total lipid (%)	3.27±0.077^{a}	3.88±0.02^{a}	4.18±0.02^{a}	4.20±0.02^{a}
Total carbohydrate (%)	40.80±0.63^{a}	39.14±0.48^{a}	39.59±0.36^{a}	40.01±0.28^{a}
Energy (kJ/100 g)	941.23±12.90^{a}	1027.02±8.9^{b*}	986.70±6.70^{a}	1068.65±4.20^{b*}

Means marked with different letters within columns for each species (male vs. female) are significantly different ($n = 3$±SD; *t*-test: $*P < 0.05$, $** P < 0.01$)

Mineral profile

In Table 2, the profiles of 14 elements in male and female pill-millipedes are compared. Of these elements, C, N, Na, K, Ca, P, Mg, and Zn were dominant (Fig. 1). Most of the elements showed significant differences among males and females. Carbon was significantly higher in males compared to females in *A. fumosa* ($P < 0.001$). Nitrogen content was significantly higher in male *A. magna* ($P < 0.01$). Ca was the most abundant among the minerals and its content was significantly higher in females than males of both species ($P < 0.001$). Females of *A. magna* contain a significantly higher amount of phosphorous and Zn than males ($P < 0.01$). K levels in female *A. fumosa* and Na levels in male *A. magna* were significantly higher. Al was found in all samples except for male *A. fumosa*. Mg, Mn and Cu contents were significantly

Table 2. Mineral profile of males and females of *Arthrosphaera* spp

Mineral (%)	*A. fumosa*		*A. magna*	
	male	female	male	female
Carbon	35.35±0.023[a]	32.98±0.48[b***]	33.18±0.56[a]	32.46±0.23[a]
Nitrogen	59.04±1.08[a]	58.24±1.23[a]	56.32±1.65[a]	48.94±0.96[b**]
Sodium	0.02±0.01[a]	0.06±0.02[a]	0.20±0.003[a]	0.03±0.001[b*]
Magnesium	0.21±0.003[a]	0.31±0.06[a]	0.36±0.01[a]	0.63±0.02[b*]
Aluminum	0.01±0.002	BDL	0.09±0.02[a]	0.01±0.003[a]
Phosphorous	0.29±0.03[a]	0.42±0.01[a]	0.63±0.008[a]	2.24±0.21[b**]
Potassium	0.07±0.009[a]	0.46±0.05[b*]	0.30±0.08[a]	0.23±0.012[a]
Calcium	4.68±0.36[a]	6.26±0.15[b***]	7.54±0.26[a]	12.73±2.56[b***]
Manganese	0.03±0.005[a]	0.05±.002[a]	0.06±0.0023[a]	0.12±0.009[b*]
Ferrous	0.01±0.001[a]	0.02±0.006[a]	0.03±0.005[a]	0.03±0.006[a]
Copper	0.20±0.009[a]	0.30±0.008[a]	0.83±0.008[a]	1.34±0.23[b*]
Zinc	0.16±0.01[a]	0.41±0.08[a]	0.27±0.02[a]	0.99±0.005[b**]
Selenium	0.01±0.003[a]	0.02±0.007[a]	0.13±0.16[a]	0.04±0.002[b*]
Lead	0.01±0.002[a]	0.18±0.001[b*]	0.03±0.002[a]	0.26±0.003[b*]
C/N ratio	0.59 ±0.02[a]	0.56 ±0.12[a]	0.58± 0.03[a]	0.66 ±0.01[a]

BDL = below detectable level. Means marked with different letters within columns for each species (male vs. female) are significantly different (n = 3±SD; t-test: *$P < 0.05$, ** $P < 0.01$,*** $P < 0.001$)

higher in female *A. magna* (P < 0.05), but Se content was higher in male *A. magna* (P < 0.05). Low Pb content was seen in both millipedes, but it was significantly higher in females (P < 0.05) than in males of *A. fumosa* and *A. magna*. There was no significant difference in C/N ratio between males and females.

Amino acid profile

A comparison of amino acids in males and females of both millipede species is presented in Table 3 (*A. magna* also in Fig. 2). The total quantity of amino acids significantly differed between males and females of *A. fumosa* (P < 0.05). The glycine was most abundant among amino acids, and its content did not differ significantly between males and females. Cysteine was the least abundant, but its level was significantly higher in females than in males (P < 0.05). The total sulphur-containing amino acids (cysteine and methionine) were significantly higher in females than in males. Serine content was significantly higher in males compared to females of *A. fumosa* (P < 0.01), while it was the opposite in *A. magna* (P > 0.05). Among aromatic amino

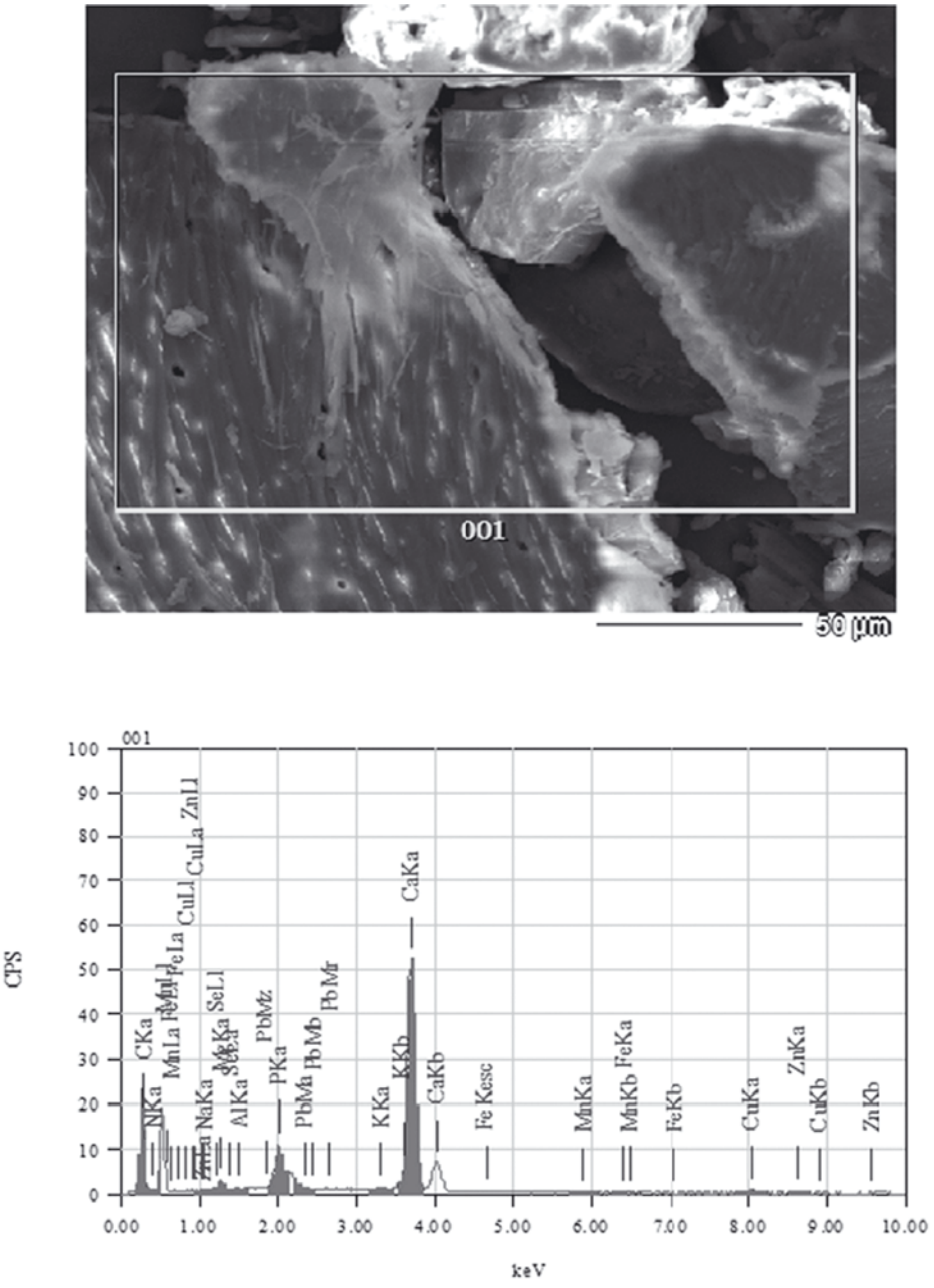

Fig. 1. Representative results of scanning electron microscope-energy dispersive spectrometry and elemental profile of female *Arthrosphaera magna* used for elemental analysis (CPS = counts per second)

Table 3. Amino acid profile of males and females of *Arthrosphaera* spp

Amino acid (mg/g)	*A. fumosa*		*A. magna*	
	male	female	male	female
Asp	21.4±0.2^{a}	352±3.7^{b**}	28.0±0.5^{a}	22.1±0.2^{a}
Glu	64.7±0.1^{a}	72.7±2.3^{a}	70.1±0.4^{a}	66.4±0.4^{a}
Ser	115.0±1.4^{a}	82.0±2.5^{b**}	58.5±0.2^{a}	78.0±0.5^{b**}
Gly	176.2±0.6^{a}	170.1±0.4^{a}	166.0±0.6^{a}	175.3±0.4^{a}
His	28.5±0.3^{a}	27.0±0.6^{a}	21.0±2.3^{a}	27.7±0.2^{a}
Arg	41.3±0.2^{a}	54.2±0.4^{b**}	51.1±0.3^{a}	47.1±0.4^{a}
Thr	40.4±0.4^{a}	40.1±0.2^{a}	37.9±0.2^{a}	42.8±0.2^{b*}
Ala	67.3±0.1^{a}	69.0±1.2^{a}	73.9±3.2^{a}	70.2±0.5^{a}
Pro	56.2±0.3^{a}	62.5±1.4^{b*}	75.9±0.3^{a}	71.8±0.3^{a}
Tyr	31.1±0.2^{a}	35.4±0.4^{b**}	48.1±0.4^{a}	40.6±0.2^{b**}
Val	48.8±0.1^{a}	50.5±0.2^{a}	54.2±0.3^{a}	55.9±0.4^{a}
Met	5.2±0.2^{a}	5.1±0.2^{a}	8.2±0.3^{a}	9.9±0.1^{b*}
Cys	0.7±0.01^{a}	2.5±0.1^{b**}	1.9±0.04^{a}	2.9±0.05^{b*}
Ile	43.1±0.4^{a}	49.4±0.2^{a}	53.0±0.5^{a}	53.5±0.4^{a}
Leu	61.3±0.3^{a}	66.3±0.4^{a}	70.8±0.4^{a}	66.4±0.1^{b**}
Phe	34.2±0.6^{a}	36.8±0.4^{a}	45.4±1.9^{a}	44.6±0.2^{a}
Lys	82.8±0.2^{a}	101.0±0.4^{b*}	102.9±0.2^{a}	84.4±1.2^{b*}
TAA	922.2±8.9^{a}	959.8±2.3^{b*}	967.4±5.6^{a}	962.6±8.7^{a}
TEAA	344.3±9.6^{a}	376.2±6.9^{b*}	393.4±8.9^{a}	385.2±6.3^{a}

TAA = total amino acids; TEAA = total essential amino acids. Means marked with different letters within columns for each species (male vs. female) are significantly different (n = 3±SD; t-test: *$P < 0.05$, ** $P < 0.01$)

acids, tyrosine level was significantly higher in male *A. magna* ($P < 0.05$), while it was significantly higher in female *A. fumosa* ($P < 0.05$). Phenyl alanine content significantly differed among males and females.

Of the non-essential amino acids, asparagine content was significantly higher in females than in males of *A. fumosa* ($P < 0.05$), while it was opposite in *A. magna* ($P < 0.05$) and no significant difference was seen in glutamic acid. On the other hand, serine was significantly more abundant in males ($P < 0.01$), proline in females ($P < 0.05$) and arginine in females ($P < 0.01$) of *A. fumosa*. Essential amino acid levels were considerably high, especially for leucine (male *A. magna*), lysine (male *A. magna* and female *A. fumosa*), histidine (female *A. fumosa*), tyrosine (male *A. magna*), and phenylalanine (male *A. magna*). The total essential amino acids were significantly higher ($P < 0.05$) in female *A. fumosa* and the opposite was true in *A. magna* ($P > 0.05$).

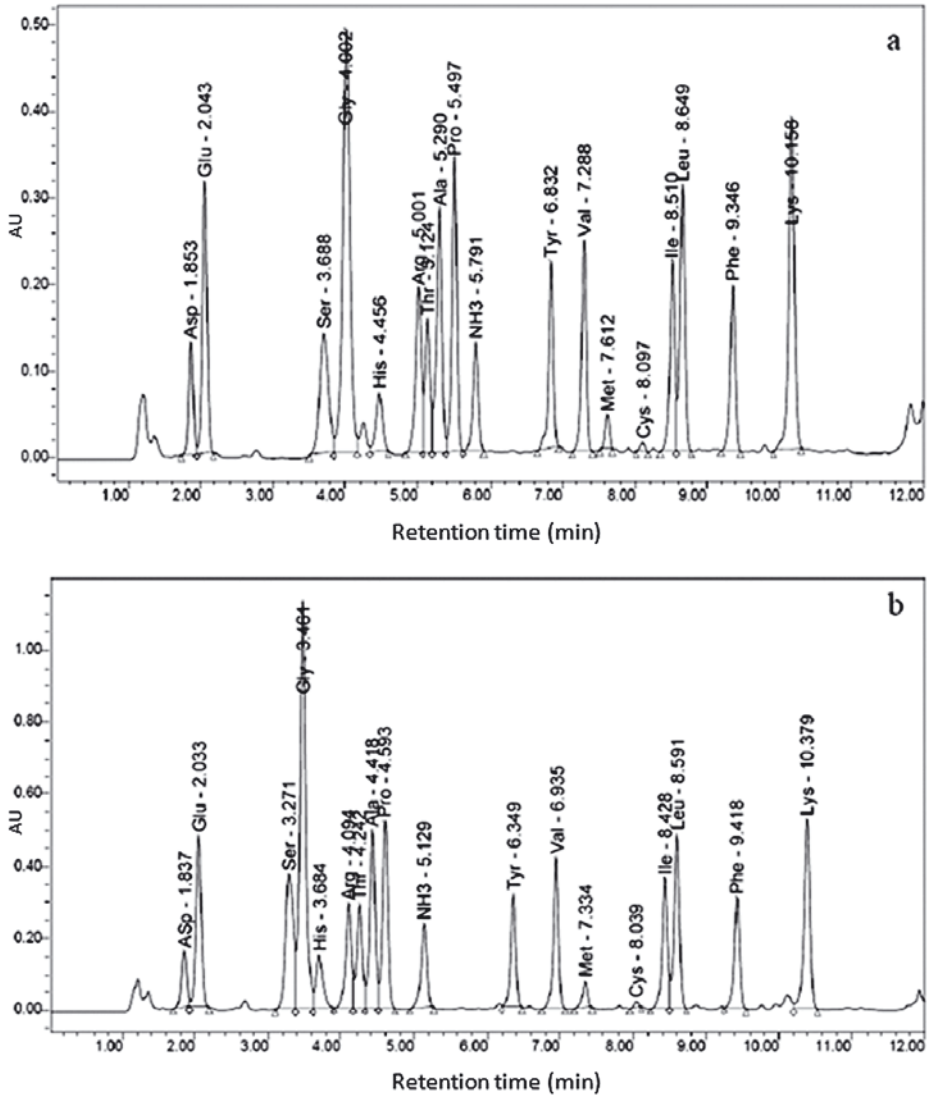

Fig. 2. Representative amino acid profiles of male (a) and female (b) *Arthrosphaera magna*, showing peaks of various amino acids (AU = arbitrary unit)

Fatty acid profile

Significant variation in several FAMEs was seen among the males and females of *A. fumosa* and *A. magna* (Table 4; *A. magna* in Fig. 3). Of the saturated fatty acids, the palmitic acid content was the highest in both sexes, which was significantly

Table 4. Fatty acid methyl esters of males and females of *Arthrosphaera* spp

Fatty acid (mg/g lipid)	*A. fumosa*		*A. magna*	
	male	female	male	female
Saturated fatty acids				
lauric acid (C12:0)	10.2±0.3^{a}	7.0±0.2^{a}	1.9±0.02^{a}	3.9±0.1^{a}
myristic acid (C14:0)	16.7±0.2^{a}	17.9±0.3^{a}	13.4±0.3^{a}	13.8±0.9^{a}
pentadecanoic acid (C15:0)	26.6±0.1^{a}	16.9±0.3^{b*}	44.1±0.9^{a}	33.7±0.6^{b*}
palmitic acid (C16:0)	163.9±1.7^{a}	129.0±0.4^{b*}	152.1±3^{a}	125.7±2.5^{b*}
heptadecanoic acid (C17:0)	46.0±0.1^{a}	36.3±0.3^{b***}	33.2±0.2^{a}	42.6±1.3^{b***}
stearic acid (C18:0)	82.0±2.3^{a}	57.3±1.2^{b*}	57.0±3.6^{a}	62.4±5.6^{a}
arachidic acid (C20:0)	20.0±0.2	BDL	5.0±0.1^{a}	5.7±0.2^{a}
heneicosanoic acid (C21:0)	BDL	BDL	1.2±0.1^{a}	1.7±0.2^{a}
behenic acid (C22:0)	11.6±0.1^{a}	7.1±0.2^{a}	5.4±0.1^{a}	4.3±0.4^{a}
lignoceric acid (C24:0)	2.8±0.5^{a}	3.1±0.6^{a}	1.7±0.1^{a}	2.1±0.1^{a}
Unsaturated fatty acids				
myristoleic acid (C14:1)	1.3±0.2^{a}	7.7±0.1^{a}	19.3±0.1^{a}	27.8±0.4^{a}
cis-10-pentadeconoic acid (C15:1)	2.6±0.4^{a}	6.2±0.2^{a}	8.6±0.3^{a}	11.2±0.6^{a}
palmitoleic acid (C16:1)	32.9±0.4^{a}	61.8±0.2^{b**}	73.4±0.6^{a}	97.0±0.1^{b**}
cis-10-heptadecenoic acid(C17:1)	BDL	14.2±0.2	15.9±0.8^{a}	19.4±0.9^{a}
oleic acid (C18:1)	311.9±2.3^{a}	376.6±8.9^{b**}	312.2±9.8^{a}	291.6±2.3^{a}
elaidic (C18:1)	20.9±5.6^{a}	8.7±0.12^{b*}	5.6±0.3^{a}	10.9±0.5^{a}
linoleic acid (18:2)	187.4±0.23^{a}	158.4±0.5^{b*}	179.8±2.3^{a}	166±8.9^{a}
linolenic (C18:3)	13.3±0.2^{a}	4.0±0.3^{b*}	3.9±0.1^{a}	3.7±0.5^{a}
gamma linolenic (C18:3)	5.6±0.1^{a}	8.1±0.1^{a}	11.8±0.4^{a}	12.2±0.5^{a}
eicosenoic acid (C20:1)	8.6±0.2^{a}	8.5±0.5^{a}	10.2±0.6^{a}	9.3±0.9^{a}
eicosadienoic acid (C20:2)	2.1±0.2	BDL	5.0±0.6^{a}	3.5±0.1^{a}
eicosatrienoic acid (C20:3)	BDL	BDL	1.5±0.1	BDL
arachidonic acid (C20:4)	11.3±0.5^{a}	6.1±0.1^{b*}	2.0±0.1^{a}	2.2±0.3^{a}
eicosapentaenoic acid (C20:5)	11.6±4.1^{a}	45.6±1.7^{b*}	25.9±2.3^{a}	31.4±4.6^{b*}
erucic acid (C22:1)	5.1±0.2^{a}	5.3±0.5^{a}	4.1±0.1^{a}	5.3±0.5^{a}
docosahexaenoic acid (C22:6)	BDL	4.0±0.8	2.1±0.6^{a}	2.4±0.1^{a}
nervonic acid (C24:1)	BDL	BDL	BDL	2.6±0.3
MUFA	383.3±3.6^{a}	489±5.9^{b*}	445.7±2.5^{a}	475.1±9.8^{b*}
PUFA	231.3±2.1^{a}	226.2±3.4^{a}	232.0±1.9^{a}	221.4±2.8^{a}
PUFA/SFA	1.6	2.6	2.1	2.3

BDL = below detectable level; MUFA = mono-unsaturated fatty acids; PUFA = poly-unsaturated fatty acids; SFA = saturated fatty acids. Means marked with different letters within columns for each species (male vs. female) are significantly different (n = 3±SD; t-test: * $P < 0.05$, ** $P < 0.01$,*** $P < 0.0001$).

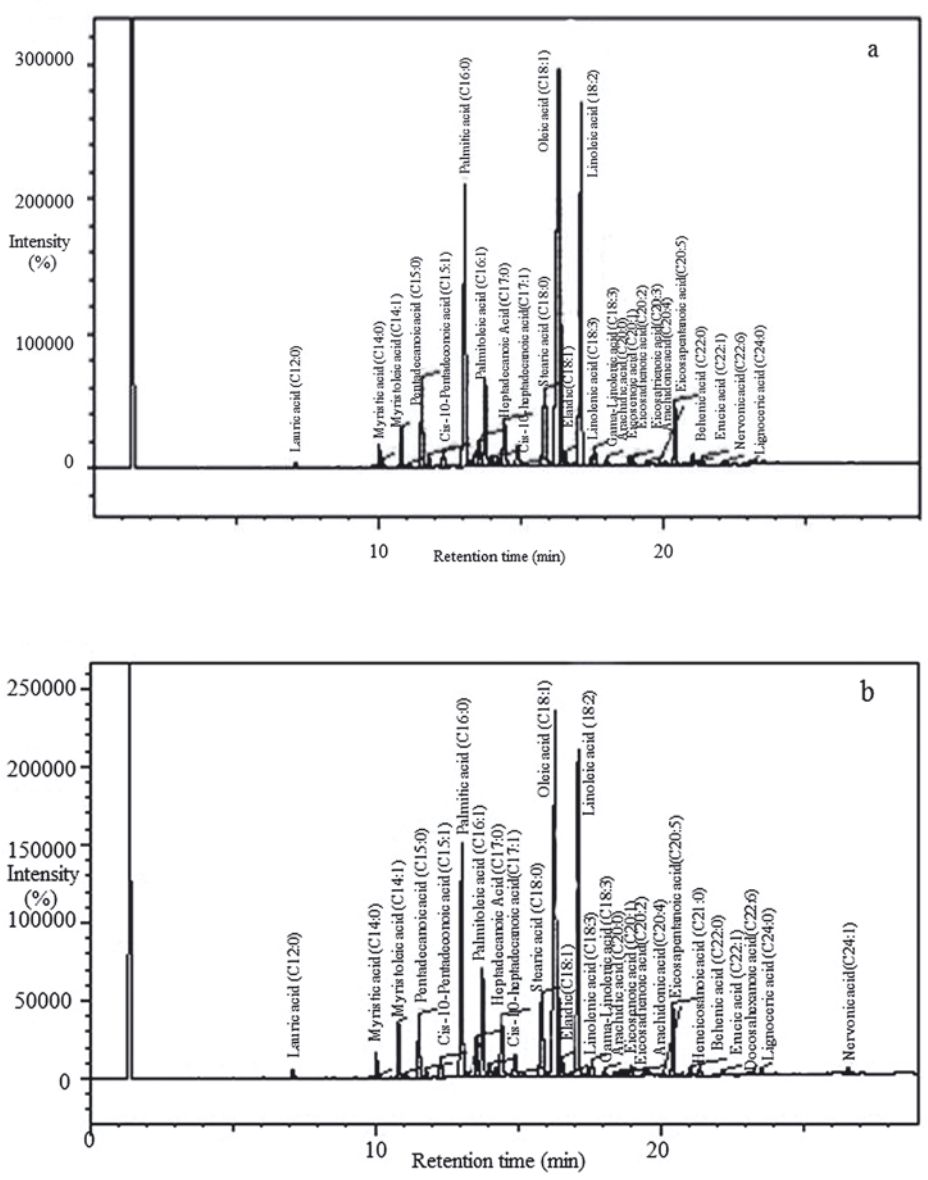

Fig. 3. Representative gas chromatograms of male (a) and female (b) *Arthrosphaera magna*, showing peaks of fatty acid methyl esters

higher in males ($P < 0.05$), followed by stearic acid (higher in male *A. fumosa* and female *A. magna*) and pentadecanoic acid (higher in males). Arachidic acid was detected in all except for female *A. fumosa*, while heneicosanoic acid was confined to *A. magna*. Heptadecanoic acid level was significantly high in male *A. fumosa* and female *A. magna* ($P < 0.001$). No significant difference was seen in behenic acid and lignoceric acid levels between sexes.

Promising quantities of unsaturated fatty acids were found in the millipedes, with the highest linoleic acid (especially high in males) followed by palmitoleic and eicosapentaenoic acids (especially high in females). The *cis*-10-heptadecanoic and docosahexaenoic acids were not detectable in male *A. fumosa*, while eicosadienoic acid, in female *A. fumosa*. Eicosatrienoic and nervonic acids were confined to male and female *A. magna*, respectively. Eicosapentaenoic acid content was significantly higher in females than in males ($P < 0.05$). Gamma linolenic acid level was higher in females than in males of *A. magna* ($P > 0.05$). Elaidic, linolenic and arachidonic acids were significantly more abundant in male than in female *A. fumosa* ($P < 0.05$). There was no significant difference in *cis*-10-pentadecenoic, myristoleic, erucic and eicosenoic acids in both millipedes. Total mono-unsaturated fatty acids (MUFA) were significantly more abundant in females than in males. The poly-unsaturated fatty acids (PUFA) did not significantly differ between the males and females. The ratio of PUFA/SFA (saturated fatty acids) was higher in females than in males.

DISCUSSION

Proximate composition

The dry mass of male and female pill-millipedes studied was comparable with those of other arthropods (Abulude & Folorunso 2003; Finke 2012). The moisture content of pill-millipedes in our study corroborates with Turkestan cockroaches (55.7-59 vs. 61.2%), Tebo worms (55.7-59 vs. 60.2%) (Finke 2012) and edible insect *Tenebrio molitor* (55.7-59 vs. 61.5%) (Ghaly & Alkoaik 2009). Ash content is higher in pill-millipedes than in other millipedes and arthropods (Abulude & Folorunso 2003; Finke 2012), reflecting its richness in minerals. The exoskeleton of pill-millipedes is a reservoir of several minerals and the high calcium content is necessary to maintain the turgidity of tergites. The crude fibre content (6.4-8.4%) of pill-millipedes was higher than that of Orthoptera (Banjo et al. 2006) and its wide variation, compared to other insects, might be due to differences in exoskeleton structures (Mariod et al. 2011).

The amount of crude protein is higher in female than in male pill-millipedes studied, but it is lower than in other millipedes (8.2-14.5% vs. 24.85%) and other arthropods (8.2-14.5% vs. 15.5-19.7%) (Abulude & Folorunso 2003; Finke 2012). The protein content of the millipede *Tymbodesmus falcatus* represents nearly 25% of its total dry mass (Enghoff et al. 2014). In the haemolymph of myriapods, protein content tends to vary between 20 and 120 mg/ml (Xylander 2009). The quantity of proteins in arthropods depends on season, moulting cycle and sex (Rajulu 1974). In female millipede *Cingalobolus bugnioni* (Spirobolida), protein content was more than 20 g of its body mass, which was about 70% higher than males (Rajulu 1974).

According to Lease & Wolf (2011), the total lipids will account for up to 4.2% in diplopods, which is consistent with the present study (3.3-4.2%). The total lipid content of pill-millipedes was higher in females than in males. Egg development in arthropods requires sufficient nutrients, so nutrient deficiency hampers the normal development of eggs (Wheeler 1996). Annual egg production in females has been correlated with fat content in fishes and lizards (Charnov et al. 2001; Warne & Charnov 2008). Studies by Lease & Wolf (2011) show that sexual dimorphism in total lipid content in many classes of arthropods (Arachnida, Chilopoda, Diplopoda, and Insecta) is likely due to the higher requirement of energy for egg production by females. Even though proteins and carbohydrates are the primary sources of energy in arthropods, the role of lipids as an energy source cannot be ruled out (O'brien 1999; Canavoso et al. 2001). The calorific value is higher for lipids, compared to glycogen, and yields more water upon oxidation (Arrese & Soulages 2010). The lipid reserves provide sufficient energy to meet the requirement for moulting, copulation, and migration in arthropods (Hahn & Denlinger 2007). An earlier study also states that ~3% of the lipids in the haemolymph of the total lipid fraction in insects (Beenakkers et al. 1985) and up to 5.1% of lipid in the Hymenoptera (Punzo 1990) meet the energy requirements. Lipids are the major constituents (15%) of the cell membrane, cuticle, and eggs of arthropods (Downer & Matthews 1976; Grapes et al. 1989). Their lipid content varies depending on physiological conditions, as it exists in different forms in the insects: as triacylglycerides, diglycerides, monoglycerides, ketone bodies, free fatty acids, phospholipids, hydrocarbons, carotenoids, and hormones (Downer & Matthews 1976).

Carbohydrates are the primary source of energy for millipedes, and the carbohydrate content of the pill-millipedes studied is higher than those of other millipedes and arthropods (Jonathan 2012). Raksakantong et al. (2010) reported that carbohydrate content of edible insects in Thailand varies from 6.7% (longan stink bug) to 16% (cicada). Another study (Jonathan 2012) showed about 20% of carbohydrates in a Thai beetle (*Heteroligus meles*). The current study also confirmed that pill-millipedes are prominent sources of carbohydrates.

The calorific value of pill-millipedes in this study is higher than in earlier observations on millipedes (Abulude & Folorunso 2003; Banjo et al. 2006; Enghoff et al. 2014). Jonathan (2012) also demonstrated a high calorific value in the yam beetle (*Heteroligus meles*) and palm weevil (*Rhynchophorus phoenicis*) in Nigeria.

Minerals

The mineral profile of the pill-millipedes studied showed that nitrogen was the most abundant element (48-59%), followed by carbon (32-35.35%) and Ca (4.7-12.7%). According to Abulude & Folorunso (2003), in millipedes usually concentrations of Ca, K, Mg and Na are high, while Zn, Fe and Mn are low. Many minerals in pill-millipedes in our study (P, K, Mn, Mg, Zn, Cu, and Se) are comparable with those of the millipede *Tymbodesmus falcatus* of Burkina Faso (Enghoff et al. 2014). The Ca content of pill-millipedes was significantly higher in females than males (6.3-12.7% vs. 4.7-7.5%). The Fe content was minimum, lower than earlier reports in millipedes (0.01% vs.1.06%) (Abulude & Folorunso 2003; Enghoff et al. 2014).

Enghoff et al. (2014) reported high levels of Ca (17.4%) and Fe (1.06%) in *T. falcatus*. Females accumulate more Ca, compared to males, as it is necessary for egg production as well as maturation. In pentatomid bugs however, Ca content was very low (*Aspongubus viduatus*: 1.02%; *Agonoscelis pubescens*: 0.76%) (Mariod et al. 2011).

Amino acids

The pill-millipedes studied possess adequate quantities of amino acids to meet the energy and metabolic requirements. This study supports the opinion of earlier investigators on insects and millipedes (Pugach & Crawford 1978; Enghoff et al. 2014). The total amino acid concentration in the haemolymph in female and male millipede *Jonespeltis splendidus* was up to 0.52 and 1.08 mg/ml, respectively (Nair & Prabhu 1971). Like in the whole body of pill-millipedes, the haemolymph of *J. splendidus* consists of lysine, cysteine, arginine, asparagine, serine, glycine, hydroxyproline, threonine, alanine, proline, tyrosine, methionine, valine, phenylalanine, leucine, and histidine (Nair & Prabhu 1971). In the millipede *Tymbodesmus falcatus*, the aromatic amino acids phenylalanine, threonine and tyrosine accounted for 1.3%, 2.5% and 1.5%, respectively (Enghoff et al. 2014), which is consistent with the present study. In *T. falcatus*, methionine, glutamic acid, and glycine were absent, while high quantities of alanine (6%) and lysine (2%) were reported. Our studies of pill-millipedes as well as an earlier study of *J. splendidus*, the major amino acids were serine and threonine (Nair & Prabhu 1971). Cysteine was high in male *J. splendidus*, but glycine and hydroxyproline were not detected in the haemolymph of the female. The sulphur-containing amino acids (methionine and cysteine) in the pentatomid bug *Aspongubus viduatus* was 57.1 mg/g crude protein, while in *Agonoscelis pubescens* it was 7.2 mg/g crude protein (Heimann 1980). These amino acids are important in cellular processes, especially oxidation/reduction (cysteine) and as a methyl donor (methionine) in metabolism (Heimann 1980). The total quantities of EAAs found in pentatomid bugs (*A. viduatus* and *A. pubescens*) were 208.5 mg/g and 119.3 mg/g crude protein, respectively, and they were lower than those of pill-millipedes.

Fatty acids

Lipids are important and have a critical role in animal metabolism (Hadley 1985). They are necessary to maintain the structural integrity of the membrane and extractable from any tissue of arthropods (Downer & Matthews 1976). Besides, lipids play a major role as energy reservoirs of tissues in arthropods necessary for migration, burrowing, and reproduction (Lease & Wolf 2011). The fatty acid profile of pill-millipedes showed an abundance of oleic acid. Palmitic and linoleic acids form the major fatty acids in males and females. Earlier studies on the millipede *Graphidostreptus tumuliporus* also showed palmitic and oleic acids up to 60-70% of fatty acids (Van Der Horst et al. 1972). Pill-millipedes possess higher amounts of PUFA than SFA, corroborating earlier studies on millipedes by Van Der Horst et al. (1972). PUFA levels were higher in males than in females, but MUFA were more abundant than PUFA in both pill-millipede species. Studies carried out on Thai dung beetles (*Helicopris bucephalus, Onthophagus mouhoti* and *O. seniculus*) also showed higher MUFA than PUFA content (Bophimai & Siri 2010). Likewise, spirostreptid

millipedes with high quantities of several essential fatty acids constitute a potential source of food for the Bobo people of Burkina Faso (Enghoff et al. 2014). The results of the present study on pill-millipedes are very similar to those of earlier studies on millipedes like *G. tumuliporus* (Van Der Horst et al. 1972) and *T. falcatus* (Enghoff et al. 2014).

CONCLUSIONS

The present study of biochemical composition of endemic giant pill-millipedes (*Arthrosphaera* spp.) of the Western Ghats of India has revealed that they are potential sources of essential biochemical constituents. The pill-millipedes in the Western Ghats are traditionally considered as having medicinal properties (for topical applications), but they appear to be also beneficial nutritionally. Both the analysed species of pill-millipedes serve as reservoirs of essential minerals, essential amino acids, and essential fatty acids. Further *in vivo* and *in vitro* studies are needed to understand the complete nutritional potential of pill-millipedes. As pill-millipedes are saprophagous and have a high potential to process plant lignocellulosic wastes, they become important for organic farming and also serve as potential medicinal and nutritional sources.

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