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Antioxidant and antibacterial properties of *Brocchia cinerea* (Vis.) and *Matricaria pubescens* (Desf.) ethyl acetate extracts and their fractions

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ABSTRACT

During this work, the polyphenols in both Asteraceae Brocchia cinerea (Vis.) and Matricaria pubescens (Desf.) were targeted by two extraction methods, using different solvents. The extracts obtained by ethyl acetate were selected for column fractionation. Indeed, four fractions are obtained for M. pubescens and five for B. cinerea. The evaluation of the antioxidant capacity of these samples was carried out by two tests, commonly used in this field. The first one uses the free radical 1, 1-diphenyl-2 - picrylhydrazyl (DPPH) while the other (FRAP) uses 2,4,6-tris-2-pyridyl-s-triazine (TPTZ). These two tests revealed the radical scavenging power and reducing potential of both species B. cinerea and M. pubescens. Results have shown that the fraction obtained with the mixture EtOH/petroleum ether at (60/40) in the case of B. cinerea and that trained by 75% methanol in dichloromethane from M. pubescens, contained the majority of their active compositions. AcOEt extracts of both species and their fractions were tested against pathogenic bacterial strains, Gram-: Pseudomonas aeruginosa ATCC 27853, Enterobacter cloacae ATCC 13047 and Gram+: Staphylococcus aureus ATCC 25923, Enterococcus feacalis ATCC 29212. M. pubescens samples have exposed particular efficacy against E. cloacae and E. feacalis with minimal inhibitory concentration (MIC) estimated at 0.125 mg/ml.

Keywords: *Matricaria pubescens*, *Brocchia cinerea*, polyphenols, flavonoïds, antioxydant, antibacterial.

INTRODUCTION

Many studies focus on the search of original natural substances such as alkaloids, phenolics, steroids, which are of great interest; as for the medical and pharmaceutical field, as for that of industries: food processing, cosmetics, etc. Among the objectives targeted by these works, those seeking natural antioxidants [1, 2], that are potent and non-toxic, capable to replacing synthetic compounds.

In addition, and following the increase in treatment failures associated with multi-resistant bacteria; effective antimicrobial agents are also sought; this has become a worldwide concern for public health. [3]

Among the highly sought natural products, appear the polyphenols of plants. They are widely used as vascular-protective [4], anti-inflammatory [5], antioxidant and antiradical [6, 7]. In addition, their microbiological activity have been proven by several scientific studies [8, 9]

Outside of often officinal conventional medicinal plants, known and used for more or less time; this area is still very fertile and it remains much to do to complete the inventory of species that could be applied in therapy or in nutrition [10].

In this context, we intend to continue our contribution to the phytochemical study and the highlighting of the biological potential of *Matricaria pubescens* (Desf.) and *Brocchia cinerea* (Vis.), which belongs to the Asteraceae family.

They are two spontaneous species, from the southern of Algeria, famous and widely used in medicine and traditional practices in this area [11]. Their richness in natural products such as phenolic compounds, terpenoids, has been reported in previous studies [12-14].

In this study, we aim the evaluation of antioxidant capacity of the fractions prepared from extracts of these two plants, as well as the checking of their antibacterial activity.

MATERIALS AND METHODS

2.1. Chemicals

1, 1-diphényl-2 - picrylhydrazyle (DPPH), Ascorbic acid, were purchased from Sigma-Aldrich (USA). 2,4,6-tris-2-pyridyl-s-triazine (TPTZ) was from Alfa Aesar (USA), silica gel 60 (particle size 230-400 mesh) from Merck. All other reagents and solvents were of analytical grade.

2.2. Plant materials

Brocchia cinerea (Vis.) and *Matricaria pubescens* (Desf.) were collected during their flowering stage (February 2016) from two localities of Algeria southeast. The first one was collected at 10 Km northern Ouargla city, the other one was brought from Still which is located at 50 Km southern Biskra city. Their botanical identification was made by O. Oulad Belkhir agronomist at the University of Ouargla. Samples were deposited at the herbarium of Biogeochemistry of desert environments laboratory at the University of Ouargla.

After drying in the shade, the plant material of *M. pubescens* was milled in power mill (diameter sieve: 4mm) then used. That of *B. cinerea* was used fresh, in the form of very small fragments cut by hand.

2.3. Preparation of crude extracts

Polyphenols in the two plants were targeted by a variety of organic solvents using two extraction methods.

II. 2.1. Case of *M. pubescens*

500g of plant powder were macerated three times (24 hx3) in a hot hydromethnolic mixture (70%). After filtration and concentration of the combined extracts, liquid-liquid extractions were carried out on the aqueous phase with ethyl acetate (AcOEt) and then with the butanol (BuOH) to obtain at the end EtOAc and BuOH crude extracts.

2.3.1. Case of *B. cinerea*

800 g of *B. cinerea*, freshly collected, were subjected to successive macerations in different organic solvents by increasing polarity. Maceration in each of these solvent (CH_2Cl_2, EtOAc, BuOH and finally EtOH) lasted 24 hours and renewal 3 times each. It is noted that before using a new solvent, the plant material must be dried from the previous one.

2.4. Fractionation of AcOEt extracts

2g of *M. pubescens* AcOEt extract were separated on silica gel, by the elution system (MeOH /CH_2Cl_2) at different proportions as follows: (25/75, 50/50, 75/25, 100/0).

Similarly, the operation was performed on 0.9 g of *B. cinerea* extract, using 200 ml of each percentage of EtOH/petroleum ether (Etp) mixture, in this order (20/80, 40/60, 60/40, 80/20, 100/0). Preliminary composition analysis, of the fractions, was monitored by bi-dimensional paper chromatography. The chromatograms were then visualized under UV lamp at 254 nm and 356 nm, before and after exposition to NH_3 vapors.

2.5. Evaluation of the antioxidant capacity

2.5.1. DPPH radical scavenging assay

The procedure used is that described by D. Huang *et al*. [15] with minor modifications.

Indeed, 3900 µl of DPPH$^{\bullet}$ solution (6.10^{-5} M), prepared in methanol, were added to 100 µl of the sample. After incubation of mixtures in the dark for 30 minutes their absorbance were measured at 517 nm. Each test was made in triplicate. The percentage of free radical DPPH scavenging (% IP) was calculated using equation (1).

$$\%PI= [(A_{cont}-A_{samp}) *100] / A_{cont}\text{……(1)}$$

Where % PI: Percentage of inhibition power, A_{cont}: absorbance of control ($DPPH^{\bullet}$ solution), A_{samp}: sample absorbance with the $DPPH^{\bullet}$ solution.

2.5.2. FRAP (Ferric Reducing Antioxidant Power) assay

This method measures the reducing power of the antioxidants present in a mixture by their ability to reduce ferric tripyridyl-triazine (Fe^{+3}-TPTZ) to ferrous tripyridyl-triazine (Fe^{+2}-TPTZ) at an acidic pH.

The FRAP assay was performed according the study [16] with minor modifications. A fresh solution of FRAP reagent was prepared by mixing 10 ml of the solution of TPTZ (10 mM) in a 40 mM HCl solution, with 10 ml of $FeCl_3.6H_2O$ (20 mM) and 100 ml of the acetate buffer solution at pH equal to 3,6. This mixture was maintained at 37 °C.

150 µl of each sample, to be tested, were reacted with 2850 µl of the FRAP reagent solution; the mixtures were incubated for 30 minutes in the dark. Thereafter, the readings of absorbance were made at 593 nm. A series of ascorbic acid concentrations was used to establish the corresponding calibration curve. The results are expressed in µg Ascorbic Acid Equivalent / mg of extract (fraction): (µg EAA/mg ext.). All experiments were made in triplicate. [15, 16]

2.6. Antibactial assay

2.6.1. Bacterial Strains

The samples prepared from *B.cinerea* and *M. pubescens* were tested against the pathogenic bacterial strains, Gram-: *Pseudomonas aeruginosa* ATCC 27853, *Enterobacter cloacae* ATCC 13047; and Gram +: *Staphylococcus aureus* ATCC 25923, *Enterococcus feacalis* ATCC 29212.

The evaluation of antibacterial activity of the different samples (AcOEt extracts and their fractions) was performed by the solid medium diffusion method [3].

2.6.2. Disk diffusion test

The various samples were solubilized in DMSO (dimethylsulfoxide) at 1 mg/ml. Paper disks of Whatman N°3 (4 mm diameter) sterilized at 120 ° C for 30 min, were soaked in 10 µl of sample solution.

In this test, Mueller Hinton was poured into Petri dishes and inoculated with a freshly prepared pure bacterial suspension. On the surface of the agar seeded, the disks were deposited. Then the whole was incubated at 37 ° C for 24 hours.

The disk containing DMSO was used as blank. Three dishes were prepared for each test.

The diameters (mm) zones of inhibition around the paper disks, allow previewing the effectiveness of extracts and their fractions against the tested germs.

For more precision, the minimum inhibitory concentration (MIC) of AcOEt extracts of *B. cinerea* and *M. pubescens* and their fractions were determined. Indeed, the same previous steps were repeated for the dilutions 1/2, 1/4, 1/8 and their stock solution (1 mg/ml) for each sample.

RESULTS AND DISCUSSION

3.1. Yields of extractions and fractionations

The extraction by AcOEt, of *M. pubescens* aerial parts, has provided a light brown extract; it has presented a return of 0.53%. The EtOAc extract obtained from *B. cinerea* was bright yellow color with a yield of 0.11%.

AcOEt extracts of both plants were found to be less profitable than the other ones. However, those obtained by butanol, equal respectively to 3.12% and 0.21%, were more important. This could mean that both species contain a significant amount of polar polyphenols such as polyglycosylated heterosides, but mono-glycosylated and di-glycosylated polyphenols, usually encountered in AcOEt extracts, were quantitatively less present in their polyphenolic composition. These results are in agreement with those in the literature [12, 14].

According to the results of preliminary TLC tests, the elution system constituted of ethanol and petroleum ether (EtOH / Etp) was chosen to fractionate *B. cinerea* AcOEt extract, whereas the one formed by MeOH and CH_2Cl_2 (MeOH/DCM)) was used with *M. pubescens* AcOEt extract. Indeed, five fractions were obtained from the first extract and four for the other one. The fractions weights are summarized in the table 1.

Table 1: Fractionation results of *B. cinerea* and *M. pubescens* AcOEt extracts

Plante		Fb1	Fb2	Fb3	Fb4	Fb5
B. cinerea	(Etp / EtOH) %	80 / 20	60 / 40	40 / 60	20 / 80	0 / 100
	masse (g)	0.1493	0.4671	0.1249	0.0531	0.0222
M. pubescens		Fm1	Fm2	Fm3	Fm4	--
	(CH_2Cl_2/ MeOH)%	75 / 25	50 / 50	25 / 75	0 / 100	--
	masse (g)	0.8831	0.8429	0.168	0.2322	--

3.2. Evaluation of antioxidant activity

3.2.1. DPPH assay

The antioxidant activity of AcOEt extracts and their fractions was evaluated using DPPH method. Ascorbic acid was used as a standard. The inhibition percentages of all the samples are shown graphically in figures 1 and 2.

In the case of *M. pubescens*, the crude AcOEt extract have shown an inhibition power (%IP) equivalent to 43.95%, that indicating a moderate free radicals scavenging potential.

The% IP recorded for the four fractions: Fm1 fractions (25%), Fm2 (50%), Fm3 (75%) and Fm4 (100%), shown in figure 1, vary between 24.84% and 70.88%.

The fraction Fm3 has proved to be the most powerful to catch DPPH free radicals with an IP% equal to 70.88%; even stronger than the AcOEt extract.

This is, probably, due to the nature of the products entrained in this fraction (flavononic structures, polyhydroxyl substitution, polarity,) and/or the synergistic effect between them. Namely, this fraction was eluted during the fractionation of the crude extract by 75% in methanol.

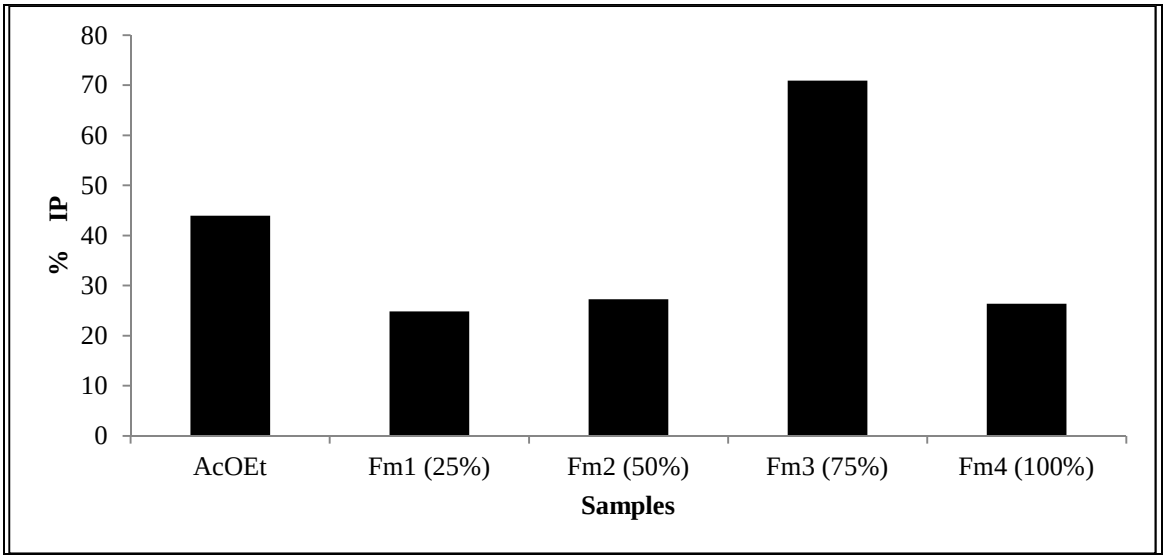

Figure 1: The percentage inhibitory power (% IP) of *M. pubescens* samples determined by DPPH assay

These findings suggest the polarity of the products responsible of the anti-radical power, in *M. pubescens* AcOEt extract. In fact, the mixture MeOH / DCM (75/25) was very effective to entail them. Purification work on this fraction might reach the active substance(s).

Regarding the inhibition percentages (% IP) of *B.cinerea* samples, they are shown in figure 2.

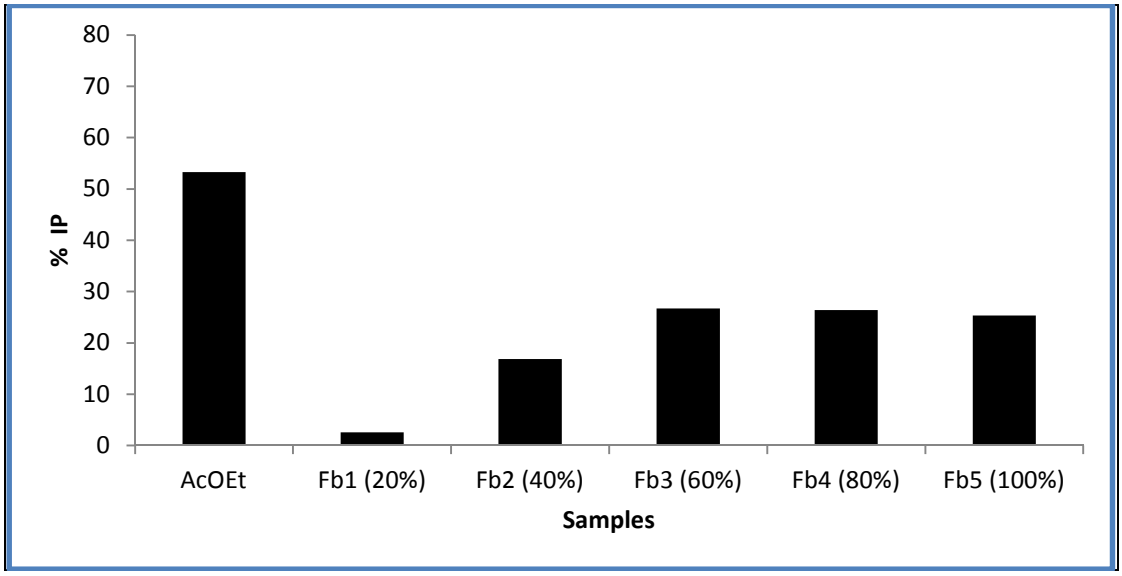

Figure 2: The percentage inhibitory power (% IP) of *B. cinerea* samples determined by DPPH assay

Overall, the samples of *B. cinerea* were more or less active. The greatest antioxidant powers are shown by AcOEt extract (53.29%) and its fractions Fb3 (60%), Fb4 (80%) and also Bb5 (100%), whose values are respectively, 26.73%, 26.38 and 25.35%.

That of the fraction Fb1 (20%) estimated at 2.58%, has showed its ineffectiveness. In fact, it was separated by a percentage in petroleum ether equal to 80%; it would probably not contain the desired active products.

On the light of these results, it is noted that the antiradical power of *B. cinerea* AcOEt extract is concentrated in the fractions Fb3, Fb4 and Fb5 that were obtained, during fractionation, with considerable percentages in EtOH equivalent respectively to 60 %, 80% and 100%.

Generally, the antioxidant activity of the tested samples is closely related to their chemical composition. Advanced studies are devoted to demonstrate the structure-activity relationship regarding antioxidant activity for the phenolic compounds in particular, flavonoids, plus the synergistic effects between these substances deemed well in this area [9].This could justify the results recorded in this study for *M. pubescens* and *B. cinerea*.

To release, the inhibition rate, from the consequences of increasing and/or decreasing the concentration of an antioxidant, the effective concentrations (EC_{50}) of the extracts and their fractions were determined. Recorded results are summarized in table 2, where most of *M. pubescens* samples have shown their efficiency, especially the significant value of Fm3 (0,73±0,002 mg/ml). Once again this fraction confirmed its appreciable content of active products.

In the case of *B. cinerea*, the calculated concentrations for the samples indicated a moderate activity for them.

Table 2: EC_{50} values determined by DPPH assay for *B. cinerea* and *M. pubescens* samples

			EC_{50} mg/ml				EC_{50} mg/ml
B. cinerea	AcOEt (brut)		0,828±0,007	***M. pubescens***	AcOEt (brut)		0,86±0,011
	(EtOH / Etp)%	Fb1(20/80)	4,059±0,007		**(MeOH / CH_2Cl_2)%**	Fm1 (25/75)	1,251±0,013
		Fb2(40/60)	2.759±0,01			Fm2 (50/50)	1,052±0,006
		Fb3(60/40)	1,605±0,002			Fm3 (75/25)	0,73±0,002
		Fb4(80/20)	1,647±0,017			Fm4 (100/0)	1,179±0,005
		Fb5(100/0)	1,706±0,01			--	--

Overall, these concentrations were more or less close to those of pure products [17-19]. However, the EC_{50} of ascorbic acid was so low; it was estimated at 47±0.034µg/ml in the same conditions.

3.2.2. FRAP assay

The concentrations equivalent to ascorbic acid, to which the samples showed reducing activity towards iron, are summarized in table 3.

Table 3: FRAP assay results of *B. cinerea* and *M. pubescens* samples

			µg EAA/mg ext.				µg EAA/mg ext.
B. cinerea	AcOEt (brut)		498,333±0,013	***M. pubescens***	AcOEt (brut)		985,333±0,013
	(EtOH / Etp)%	Fb1(20/80)	57,333±0,001		**(MeOH / CH_2Cl_2)%**	Fm1 (25/75)	722,666±0,025
		Fb2(40/60)	476,333±0,007			Fm2 (50/50)	759.32±0,016
		Fb3(60/40)	1006,666±0,503			Fm3 (75/25)	821,333±0,008
		Fb4(80/20)	608,666±0,304			Fm4 (100/0)	368,333±0,040
		Fb5(100/0)	547,333±0,008			--	-- --

Reading FRAP test results reveal that *M. pubescens* AcOEt extract possesses an interesting power to reduce iron; it was estimated to 985.333±0.013 µg EAA/mg ext. Moreover, the values calculated for its three first fractions were very significant; they vary between 722.666±0.025 and 821.333±0.008 µg EAA/mg ext. However, the last one Fm4 (100%) appeared less reducer, compared to previous fractions, with a concentration evaluated to 368.333 ± 0.040 µg EAA/mg ext.

According to these findings, AcOEt extract seems be very rich in products, capable of reducing iron, such as polyphenols and their derivatives including flavonoids, which may be present in this sample and its fractions, particularly Fm3 (75%).

Therefore, *M. pubescens* includes a significant reduction potential, it can be used as a natural source of antioxidants.

In the case of *B. cinerea*, AcOEt extract has presented an acceptable reducing power towards metals; it's equivalent ascorbic acid concentration was equal to 498.333±0.013 µg EAA/mg ext.

Except the first fraction Fb1 (20%), which showed a low value (57.333±0.001 µg EAA/mg ext.); the other fractions revealed a significant reduction potential. This was translated by the most notable value of Fb3 (1006.666±0.503 µg EAA/mg ext) plus those recorded for Fb4 (80%) and Fb5 (100%), respectively equivalent to 608.666±0.304 and 547.333±0.008 µg EAA/mg ext.

These results show clearly, the antagonistic effect between the constituents of *B. cinerea* EtOAc extract, or some of them, when they are together.

However, their separation into fractions, according to the used eluting system, has exhibited significant synergistic effects between one fraction products, such as the significant action of compounds present in Fb3.

The found concentrations testify that the plant *B. cinerea* contains very active compounds (supposed to be polyphenolics), with different polarities and interesting structures. These products give it, its importance as a natural source of antioxidants.

3. 3. Antibacterial assay

The minimum inhibitory concentrations (MIC) determined for AcOEt extracts and their fractions of both plants, are summarized in table 4. This one illustrates the variability of the MIC according to the tested bacteria: *E. cloacae, E. feacalis, P. aeruginosa and S. aureus*.

In general, most of the tested extracts exhibited considerable MIC. They ranged between 0.125-0.5 mg/ml.

E. cloacae, E. feacalis, have shown an interesting sensitivity to all tested samples, especially that exhibited by *M. pubescens* fractions against the first germs.

These findings allow reporting that the antibacterial effect of tested extracts varies nearly, with their composition in polyphenols and/or flavonoids, known by their antibacterial effects [20, 21] [8].

Furthermore, AcOEt extract of *B. cinerea* was more effective against *S. aureus* and *P. aeruginosa*, with MIC equal to 0.25 mg/ml, compared to *M. pubescens* extract, or even its fractions.

By performing these tests, the variability of the antimicrobial property was expected, because microbial growth inhibition depends upon several factors such as used germs and the tested extracts as well as the mechanisms of action of these ones. [22]

Table 4: The minimum inhibitory concentrations results of *B. cinerea* and *M. pubescens* samples

	Extraits et fractions			*S. aureus* Gram +	*P. aeruginosa* Gram -	*E. feacalis* Gram +	*E. cloacae* Gram -
B. cinerea	AcOEt (brut)		**CMI (mg/ml)**	0.25	0.25	0.125	1
	(EtOH / Etp)%	20/80		1	1	0.25	0.25
		40/60		0.5	1	0.5	0.5
		60/40		0.5	1	0.5	0.5
		80/20		0.25	0.5	0.125	0.125
		100/0		--	--	--	--
M. pubescens	AcOEt (brut)		**CMI (mg/ml)**	1	1	0.125	0.125
	($MeOH/CH_2Cl_2$)%	25/75		--	--	0.125	0.125
		50/50		1	--	--	0.125
		75/25		--	0.5	0.125	0.125
		100/0		1	1	0.5	0.25

Fractionation system: ($MeOH/CH_2Cl_2$)%, (EtOH/Petroleum ether)%, CMI (mg/ml) : concentration minimale inhibitrice (mg/m).

In comparison with studies performed on natural extracts [23, 24], the MIC determined in our work reveal the significant antibacterial potential of *B.cinerea* and *M. pubescens* especially, against *E. cloacae* and *E. feacalis*.

It should be noted that the inhibitory action of samples, on tested germs, is lower than that of standard antibiotics. However, the positive results of most of our samples, used as mixtures [25], are very informative about the presence of effective antibacterial agents among the composition of some fractions compared to other, as shown in table 4.

CONCLUSION

The findings of the present research, allow concluding that *Brocchia cinerea* (Vis.) includes an important reducing potential, whose compounds could be reached in its polar fractions. Regarding *Matricaria pubescens* (Desf.), it possesses a very interesting antiradical chemical composition, which can be extracted firstly, by ethyl acetate and then will be trained by the mixture constituted of 75% methanol and 25% dichloromethan. Also, both species have shown their positive antimicrobial activity against *E. cloacae* and *E. feacalis* that indicate the presence of antibacterial agents in their ethyl acetate extracts. Further purification works on these active fractions will be required to find the active compound(s); that might help scientists and economists to resolve serious nutrition and health problems.

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