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SCREENING OF POLYPHENOLIC COMPOUNDS, ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF *TANACETUM VULGARE* FROM TRANSYLVANIA

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ABSTRACT. The purpose of this study was to investigate the phenolic compounds and to evaluate the antioxidant and antimicrobial properties of *Tanacetum vulgare* flowers from different areas of Transylvania (Romania). The identification and quantification of major phenolic compounds were performed by a LC-MS method. The total polyphenols, caffeic acid derivatives and flavonoids content was spectrophotometrically determined. The antioxidant activity was evaluated using the DPPH bleaching method. The antimicrobial tests were performed using the disk diffusion assay. The phenolic profile showed the presence of phenolic acid derivatives (gentisic, caffeic, chlorogenic, *p*-coumaric, and ferulic acids), flavonoid glycosides (hyperoside, isoquercitrin, rutin and quercitrin) and flavonoid aglycones (quercetin, patuletin, luteolin, kaempferol, apigenin). The extracts of *T. vulgare* from Sibiu contain higher amounts of total polyphenols, flavonoids and caffeic acid derivatives than the extracts of *T. vulgare* from Alba. Both ethanolic extracts showed good antioxidant activity and low inhibitory activity against Gram-positive bacteria. The displayed potent antioxidant activity of these extracts supports the ethnomedicinal uses for this species.

Keywords: *Tanacetum vulgare, HPLC-MS, polyphenols, antioxidant, antimicrobial activities*

INTRODUCTION

Tanacetum vulgare L. syn. *Chrysanthemum vulgare* (L.) Bernh., commonly known as tansy, is a species of the genus *Tanacetum* from *Asteraceae* family [1,2].

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The flora of Romania comprises around 12 species and a few subspecies and varieties of *Tanacetum* [2]. Some members of this genus are very important species for their medicinal value used over the years in all around the world. *T. vulgare* has a long history of medicinal use. In traditional medicine, tansy is used to heal wounds, fever, headache, gastrointestinal and liver diseases, rheumatic pain, and intestinal parasites, due to anthelmintic, carminative, antiseptic, antihypertensive, diuretic, anti-inflammatory and antispasmodic properties [1,3-6]. Previous studies on T*. vulgare* from France showed antioxidant and antiviral properties against the herpes simplex viruses HSV-1 and HSV-2 [7-8]. The crude alcoholic extract and the essential oil from *T. vulgare* possess vermifuge activities [9]. Phytochemical studies have shown that *T. vulgare* contain several biologically active metabolites, mainly sesquiterpene lactones, essential oil, phenolic compounds, polysaccharides [1,3,8,10-13]. Since no data on chemical composition of *T. vulgare* flowers from Romania was found in the literature, the aim of this study was to carry out a phytochemical analysis of this natural product, and to investigate the antioxidant and antimicrobial properties.

RESULTS AND DISCUSSION

Identification and quantification of phenolic compounds

Because of the complexity of the mixtures of plyphenolic compounds in the natural plant extracts, it is rather difficult to characterize every compound, but it is important to identify the major groups and the types of phenolic compounds [14]. In this work, the major types and their representative constituents of phenolic compounds in *T. vulgare* extracts were determined by HPLC analysis. In this regard, a liquid chromatographic method for the determination of nineteen phenolic compounds (eight phenolic acids, four quercetin glycosides, and seven flavonol and flavone aglycones) from natural products was used. The simultaneous analysis of different classes of polyphenols was performed by a single column pass, and the separation of all examined compounds was carried out in 35 min. The concentrations of identified polyphenolic compounds in the two samples of *T. vulgare* are presented in Table 1 and the HPLC chromatograms are shown in Figures 1-2. Quantitation was performed according to an external standard method [15-19].

Chlorogenic acid was identified in the two ethanolic extracts. *T. vulgare* from Sibiu was richer in chlorogenic acid (4334.32 ± 11.79 µg/g), than the sample harvested from Alba (3673.75 ± 15.70 µg/g) (Table 1). Caffeic acid and gentisic acid were identified in the extract of *T. vulgare* (Sibiu), but their concentration was too low to be quantified. Ferulic acid and *p*-coumaric acid

were found only in the sample from Alba (3.55 ± 0.32 and 4.41 ± 0.08 µg/g respectively). The pattern of flavonoids indicates large quantitative differences between the two samples. Rutin and quercitrin were quantified in higher amounts in *T. vulgare* (Sibiu) *versus T. vulgare* (Alba). Thus, rutin was found in very large quantities in *T. vulgare* from Sibiu (350.15 ± 7.30 µg/g), whereas, in the sample from Alba, the content of rutin was below than 0.2 µg/g. The amount of quercitrin was 8.62 times higher in the sample from Sibiu, than the sample collected from Alba County (112.09 ± 2.91µg/g, and 13.00 ± 0.04 µg/g, respectively). Hyperoside and isoquercitrin were identified and determined in small amounts in both samples (Table 1). Five flavonoid aglycones, i.e. quercetin, patuletin, luteolin, kaempferol and apigenin were found in the extracts of *T. vulgare*. Luteolin and quercetin were found in large quantities in both extracts (Table 1). Apigenin was quantified only in the sample from Sibiu, while kaempferol was identified only in the tansy of Alba (below limit of detection). Patuletin was also identified in the two extracts, but in too low concentration to be quantified. According to various literature sources, apigenin, acacetin, luteolin, cinaroside, eupatilin, cosmosiin, tilianin and jaceoside are the main flavonoids in tansy flowers [10-11].

Table 1. Polyphenolic compounds content in *T. vulgare* extracts (µg/g plant)

Polyphenolic compounds	**Rt ± SD (min)**	***m/z***	***T. vulgare* (Sibiu)**	***T. vulgare* (Alba)**
Gentisic acid	2.15 ± 0.07	179	<0.2	-
Caffeic acid	5.60 ± 0.04	179	<0.2	-
Chlorogenic acid	5.62 ± 0.05	353	4334.32 ± 11.79	3673.75 ± 15.70
p-Coumaric acid	8.70 ± 0.08	163	-	4.41 ± 0.08
Ferulic acid	12.20 ± 0.10	193	-	3.55 ± 0.32
Hyperoside	18.60 ± 0.12	463	17.51 ± 0.50	23.73 ± 0.37
Isoquercitrin	19.60 ± 0.10	463	14.29 ± 0.22	18.91 ± 0.10
Rutin	20.20 ± 0.15	609	350.15 ± 7.30	<0.2
Quercitrin	23.64 ± 0.13	447	112.09 ± 2.91	13.00 ± 0.04
Quercetin	26.80 ± 0.15	301	69.45 ± 2.86	56.24 ± 1.22
Patuletin	29.41 ± 0.12	331	<0.2	<0.2
Luteolin	29.10 ± 0.19	285	122.21 ± 2.91	165.77 ± 2.24
Kaempferol	32.48 ± 0.17	285	-	<0.2
Apigenin	33.10 ± 0.15	279	20.50 ± 1.60	-

Note: NF - not found, below limit of detection. Values are the mean ± SD (n = 3).

Considering the 19 standard compounds used in this study, some other peaks were not identified. We analyzed the polyphenolic composition of the two ethanolic extracts of *T. vulgare* collected from different areas. The

simultaneous determination of wide range of polyphenolic compounds was performed using a rapid, highly accurate and sensitive HPLC method assisted by mass spectrometry detection [15-19]. The polyphenolic profile showed the presence of phenolic acid derivatives, four flavonoid glycosides and five flavonoid aglycones in *T. vulgare* flowers (Table 1, Fig. 1, 2). Additionally, the obtained results showed that these samples of *Tanacetum* could be considered as a *good* source of chlorogenic acid.

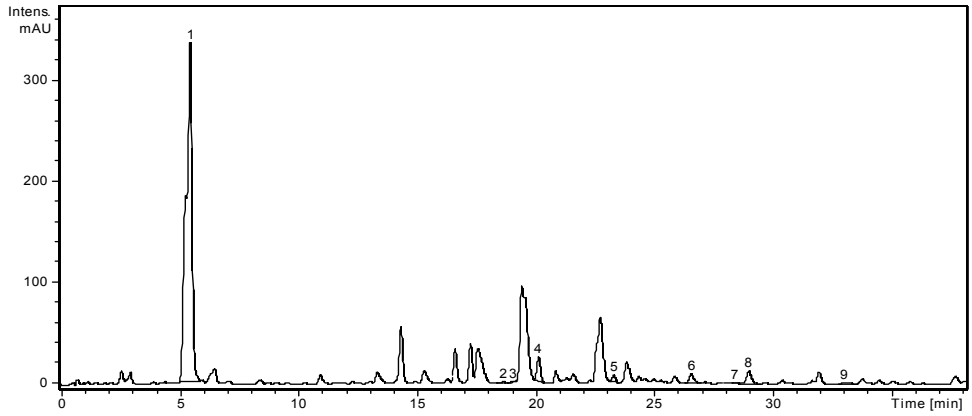

Figure 1. HPLC chromatogram of *T. vulgare* extract (Sibiu)
Notes: The identified compounds: 1, Chlorogenic acid; 2, Hyperoside; 3, Isoquercitrin; 4, Rutin; 5, Quercitrin; 6, Quercetin; 7, Patuletin; 8, Luteolin; 9, Apigenin

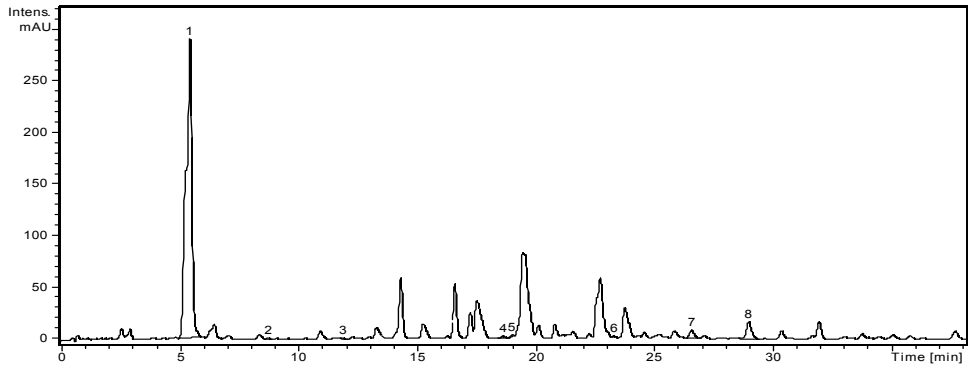

Figure 2. HPLC chromatogram of *T. vulgare* extract (Alba)
Notes: The identified compounds: 1, Chlorogenic acid; 2, p-Coumaric acid; 3, Ferulic acid; 4, Hyperoside; 5, Isoquercitrin; 6, Quercitrin; 7, Quercetin; 8, Luteolin

Estimation of polyphenol content and the antioxidant activity

The polyphenolic compounds, particularly flavonoids, have attracted a great deal of research on their broad distribution in medicinal plants, their physiological (including antioxidant) activities, and their health effects. While the flavonoid composition of many species from *Asteraceae* family was already determined, less is known about their phenolic acid derivatives and total polyphenolic content [8]. Due to their importance in plants and human health, it would be useful to know the concentration of the polyphenolic compounds which could indicate their potentials as therapeutic agents, but also for predicting and controlling the quality of medicinal herbs [16]. Thus, we determined the content of polyphenols in *T. vulgare* flowers from different areas of Romania. Also, we investigated the total antioxidant capacity, determined *in vitro* using the 2,2'-diphenyl-1-picrylhydrazyl free radical (DPPH) assay [17,20]. Table 2 gives comparative quantitative data on polyphenolic compounds levels in the two ethanolic extracts of *T. vulgare* from Sibiu and *T. vulgare* from Alba.

Concerning the content of total polyphenols, the highest amounts were determined in the extracts of *T. vulgare* collected from Sibiu (50.06 ± 3.42 mg/g) and *T. vulgare* from Alba (46.75 ± 3.37 mg/g) (Table 2). Also, the samples from Sibiu are richer in flavonoids and caffeic acid derivatives (10.35 ± 0.73, and 11.82 ± 1.06 mg/g, respectively) than the samples from Alba (7.73 ± 0.27, and 7.70 ± 0.22 mg/g, respectively). The ethanolic extracts obtained from Romanian species contain higher amounts of total polyphenols (between 46.75 and 50.06 mg/g) than the methanolic extract of the Polish species (between 24 and 39 mg/g), as previously published by Wegiera et al. [21]. The same Polish species had a lower concentration of flavonoids (0.71 mg/g) [21] than *T. vulgare* from Romania (between 7.73 and 10.35 mg/g).

The ethanolic extracts of *T. vulgare* harvested from Sibiu and Alba were investigated for the antioxidant activity**.** The antioxidant activity was assessed by the DPPH radical bleaching method. Trolox (0.025 mg/mL) and quercetin (10µg/mL) were used as the positive controls (Table 2). The $IC_{50(DPPH\bullet)}$ values of the extracts increased in the following order: *T. vulgare* (Alba) > *T. vulgare* (Sibiu). The lower the IC_{50} value means the more powerful the antioxidant capacity. According to this method, *T. vulgare* from Sibiu, with IC_{50} = 68.74 ± 4.27 µg/mL, showed a slightly higher radical scavenging activity than *T. vulgare* from Alba (70.67 ± 4.33 µg/mL), but lower that of the standard quercetin (5.47 ± 0.16 µg/mL), or trolox (11.20 ± 0.20 µg/mL). The results were in agreement with the phenolic compounds values listed in Table 2. Therefore, it is likely that the phenolic constituents present in this species are responsible for the antioxidant and free radical scavenging activities. The literature data on the antioxidant activities of this species are difficult to compare because of the different used

methods. However, the related literature shows that this species has a good antioxidant activity [1,8,22]. Comparing the antioxidant activities of *T. vulgare* from Hadlow, U.K. and the same species from Romania, the ethanolic extracts of the Romanian species (68.74 - 70.67 µg/mL) exhibited a lower antioxidant activity than the methanol extract of the English species (37 ± 1.2 µg/mL) [22]. The displayed potent antioxidant activity of *T. vulgare* extract could support the traditional medicinal uses of the plant as wound healing, to treat rheumatic pain and other inflammatory conditions [1].

Table 2. Total phenols and the results of DPPH method for *Tanacetum* extracts

Samples	TPC (mg GAE/g)	Flavonoids (mg RE/g)	Caffeic acid derivatives (mg CAE/g)	IC_{50} (µg/mL)
T. vulgare (Sibiu)	50.06 ± 3.42	10.35 ± 0.73	11.82 ± 1.06	68.74 ± 4.27
T. vulgare (Alba)	46.75 ± 3.37	7.73 ± 0.27	7.70 ± 0.22	70.67 ± 4.33
Quercetin	-	-	-	5.47 ± 0.16
Trolox	-	-	-	11.20 ± 0.20

Each value is the mean ± SD of three independent measurements. GAE: Gallic acid equivalents; RE: rutin equivalents; CAE: caffeic acid equivalents

In vitro antimicrobial activity

Two ethanolic extracts of *T. vulgare* were investigated for their *in vitro* antimicrobial properties using a disk-diffusion method against a panel of microorganisms including *S. aureus, B. subtilis, E. coli, P. aeruginosa* and *C. albicans.* After incubation, all plates were examined for any zones of growth inhibition, and the diameter of these zones were measured in millimeters (Table 3) [20, 23].

Table 3. Results of the antimicrobial activity of *T. vulgare* extracts

Samples	Zone of inhibition (mm)				
	S. aureus	*B. subtilis*	*E. coli*	*P. aeruginosa*	*C. albicans*
T. vulgare (Sibiu)	11 ± 1.00	10 ± 0.00	6 ± 1.00	6 ± 0.00	6 ± 0.00
T. vulgare (Alba)	12 ± 1.00	11 ± 1.00	7 ± 1.00	6 ± 0.00	6 ± 0.00
Ciprofloxacin	30 ± 3.00	23 ± 1.00	36 ± 4.00	20 ± 1.00	-
Fluconazole	-	-	-	-	25 ± 2.00

Notes: The values represent the average of three determinations ± standard deviations. Ciprofloxacin (25 µg/well) and Fluconazole (25 µg /well) were used as positive control.

The investigated extracts were less active against *S. aureus* and *B. subtilis* (inhibition diameter between 10 and 12 mm) and they did not show antibacterial activity against the tested Gram-negative bacterial strains: *E. coli* and *P. aeruginosa* (inhibition diameter < 10 mm) (Table 3). The samples were also inactive on *C. albicans* (inhibition diameter < 10 mm).

The results of the present investigation suggest that *T. vulgare* ethanolic extracts exhibited a low antibacterial activity against two Gram-positive bacterial strains (*S. aureus* and *B. subtilis*). No scientific data was available regarding the antibacterial action of *T. vulgare* flowers extracts. Antimicrobial activity has been reported only for essential oil isolated from *T. vulgare* [1].

CONCLUSIONS

We have determined the polyphenolic composition and the antioxidant activity for *Tanacetum vulgare* collected from different areals of Romania, for a better pharmacognostical characterisation of the indigenous species. The antioxidant activity evaluated using the DPPH bleaching method indicated that *T. vulgare* from Sibiu has important antioxidant effect, related with the total phenolic content. The phytochemical comparative study showed minor qualitative and quantitative differences between the studied samples. The antimicrobial tests underlined a low activity against the tested Gram-positive bacterial strains. Our results show that *T. vulgare* may be considered a potential source of polyphenols with antioxidant properties. Further *in vivo* studies concerning the pharmacologic properties of this medicinal plant is required in order to obtain natural pharmaceuticals with therapeutic efficacy.

EXPERIMENTAL SECTION

Plant material and extraction procedure

The flowers of *Tanacetum vulgare* L. were collected in July 2013 from different areas of Transylvania: Sibiu County (Voucher No. 24) and Alba County (Voucher No. 25), in the blossom period. Voucher specimens were deposited in the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy Cluj-Napoca, Romania. The natural product (flowers) was reduced to a proper degree of fineness. 10.0 g of the plant material was extracted with 100 mL of 70% ethanol (Merck, Darmstadt, Germany), for 30 minutes on a water bath, at 60 °C. The samples were then cooled down and centrifuged at 4.500 rpm for 15 min. and the supernatant was recovered [15-18].

HPLC–MS analysis

An Agilent 1100 HPLC Series system (Agilent, USA) was used equipped with a degasser, binary gradient pump, column thermostat, autosampler, and UV detector. The HPLC system was coupled with an Agilent 1100 mass spectrometer (LC/MSD Ion Trap VL). For the separation, a reverse-phase analytical column was employed (Zorbax SB-C18, 100 x 3.0 mm i.d., 3.5 µm particle); the work temperature was 48°C. The detection of the compounds was performed on both UV and MS mode. The UV detector was set at 330 nm until 17.5 min, then at 370 nm. The MS system operated using an electrospray ion source in negative mode. The chromatographic data were processed using ChemStation and DataAnalysis software from Agilent, USA. The mobile phase was a binary gradient: methanol and acetic acid 0.1% (v/v). The elution started with a linear gradient, beginning with 5% methanol and ending at 42% methanol, for 35 minutes; then 42% methanol for the next 3 minutes. The flow rate was 1 mL/min and the injection volume was 5 µL [15-19]. The MS signal was used only for qualitative analysis based on specific mass spectra of each polyphenol. The MS spectra obtained from a standard solution of polyphenols were integrated in a mass spectra library. Later, the MS traces/spectra of the analyzed samples were compared to spectra from library, which allows positive identification of compounds, based on spectral mach. The UV trace was used for quantification of the identified compounds from MS detection. Using the chromatographic conditions described above, the polyphenols eluted in less than 35 minutes. Four polyphenols could not be quantified in current chromatographic conditions due overlapping (caftaric acid with gentisic acid and caffeic acid with chlorogenic acid). However, all four compounds can be selectively identified in MS detection (qualitative analysis) based on differences between their molecular mass and MS spectra. The detection limits were calculated as minimal concentration producing a reproductive peak with a signal-to-noise ratio greater than three. Quantitative determinations were performed using an external standard method. Calibration curves in the 0.5-50 mg/mL range with good linearity (R^2>0.999) for a five point plot were used to determine the concentration of polyphenols in plant samples [15-19]. The detection and quantification of polyphenols was performed in UV assisted by mass spectrometry detection. Due to peak overlapping, four polyphenol-carboxylic acids (caftaric, gentisic, caffeic, chlorogenic) were determined only based on MS spectra, whereas for the rest of the compounds the linearity of the calibration curves was very good (R^2 > 0.998), with detection limits in the range of 18 to 92 ng/mL. The detection limits were calculated as the minimal concentration yielding a reproducible peak with a signal-to-noise ratio greater than three. Quantitative determinations were performed using an external

standard method; retention times were determined with a standard deviation ranging from 0.04 to 0.19 min. For all compounds, the accuracy was between 94.1.3% and 105.3%. Accuracy was checked by spiking samples with a solution containing each standard compound in a 10 µg/mL concentration. In all analysed samples the compounds were identified by comparison of their retention times and recorded electrospray mass spectra with those of standards in the same chromatographic conditions.

Determination of polyphenolic compounds content

The total caffeic acid derivatives content was expressed as caffeic acid as previously described in the European Pharmacopoeia [24]. The extract (1 mL) was added to 2 mL 0.5 N HCl, 2 mL Arnow's reagent (10 g sodium nitrite and 10 g sodium molybdate made up to 100 mL with distilled water), 2 mL NaOH (at a concentration of 1 N) and 3 mL of distilled water. Each solution was compared with the same mixture without Arnow's reagent. The absorbance was spectrophotometrically determined at 525 nm. The percentage of phenolic acids, expressed as caffeic acid equivalent on dry weight, was calculated using an equation obtained from calibration curve of caffeic acid graph (R^2= 0.994) [16,24].

The total flavonoids content was determined and expressed as rutin, as previously described in the Romanian Pharmacopoeia [18]. Each extract (5 mL) was mixed with sodium acetate (5.0 mL, 100 g/L), aluminum chloride (3.0 mL, 25 g/L) and filled up to 25 mL with methanol in a calibrated flask. Each solution was compared with the same mixture without reagent. The absorbance was measured at 430 nm [18]. The total flavonoids concentrations were determined using an equation obtained from calibration curve of the rutin graph (R^2 = 0.999).

The total polyphenolic content (TPC) of the extracts was determined by spectrophotometric method using Folin-Ciocalteu reagent and gallic acid as standard to produce the calibration curve [17,24]. 2 mL of ethanolic extracts diluted 25 times were mixed with 1.0 mL of Folin-Ciocalteu reagent, 10.0 mL of distilled water and diluted to 25.0 mL with a 290 g/L solution of sodium carbonate. The samples were incubated in the dark for 30 min. The absorbance was measured at 760 nm and the total phenols were expressed as gallic acid equivalents/g of dry plant material (mg GAE/ g plant material) [17,24].

DPPH bleaching assay

A DPPH assay was employed to investigate the antioxidant activity of the ethanolic extracts of *T. vulgare*. The DPPH solution (0.25 mM) in methanol was prepared and 2.0 ml of this solution was added to 2.0 ml of extract solution (or standard) in ethanol at different concentrations (12.5-100 µg/mL). After 30

minutes of incubation at 40°C in a thermostatic bath, the decrease in the absorbance (n = 3) was measured at 517 nm. The percent DPPH scavenging ability was calculated as: DPPH scavenging ability = ($A_{control} - A_{sample}/A_{control}$) × 100, where $Abs_{control}$ is the absorbance of DPPH radical and methanol (containing all reagents except the sample) and Abs_{sample} is the absorbance of DPPH radical and sample extract. Afterwards, a curve of % DPPH scavenging capacity *versus* concentration was plotted and IC_{50} values were calculated. IC_{50} denotes the concentration of sample required to scavenge 50% of DPPH free radicals. Trolox and quercetin were used as positive control [16-17, 20,25-26].

Determination of antimicrobial activity

The disc-diffusion assay was used to determine the antimicrobial activity of the investigated ethanolic extracts of *T. vulgare* against a panel of microorganisms including gram-positive and gram-negative bacteria: *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis, Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) and the fungus *Candida albicans (*ATCC10231*)*[23]. Each microorganism was suspended in Mueller Hinton (MH) broth and diluted approximately to 10E6 colony forming unit (cfu)/mL. They were “flood-inoculated” onto the surface of MH agar and MH Dextroxe Agar (MDA) and then dried. Six millimetres diameter wells were cut from the agar using a sterile cork-borer and 15 µL of each extract were delivered into the wells. The plates were incubated at 37°C and the diameters of the growth inhibition zones were measured after 18 h. Ciprofloxacin (25 µg/well) and Fluconazole (25 µg/well) were used as standard drugs. The controls were performed with only sterile broth and with only overnight culture and 10 µL of 70% ethanol [23]. All tests were performed in triplicate, and clear halos greater than 10 mm were considered as positive results.

Statistical analysis

All the samples were analysed in triplicate; the average and the relative SD were calculated using the Excel software package.

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