https://doi.org/10.31925/farmacia.2019.6.22 **ORIGINAL ARTICLE**

CHROMATOGRAPHIC ANALYSIS AND ANTIBACTERIAL POTENTIAL OF EXTRACTS OF *GNETUM AFRICANUM*

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Abstract

Traditionally, *Gnetum africanum* is being used widely for its nutritional value. In the present study, extracts of these plants were evaluated for their antimicrobial activity against some human pathogenic bacteria *viz. S. aureus*, *B. cereus*, *E. coli*, *Klebsiella spp. and Enterobacter aerogenes*. Gas-chromatographic results evidenced the presence of various bio-active compounds and the extracts applied on human pathogenic bacteria presented antibacterial effect, depending on the type of solvent used for the extraction and also the bacterial strain of concern, exception was made by the chloroformic extract which did not exhibit any antibacterial effect on the bacterial strains selected in the study.

Rezumat

În mod tradițional, *Gnetum africanum* este utilizat pe scară largă pentru valoarea sa nutritivă. În studiul de față, extracte din aceasta planta au fost evaluate pentru activitatea lor antimicrobiană împotriva unor bacterii patogene umane, si anume, *S. aureus, B. cereus, E. coli, Klebsiella spp.* și *Enterobacter aerogene.* Rezultatele cromatografice au evidențiat prezența diferiților compuși bioactivi, iar extractele aplicate pe bacteriile patogene umane au prezentat efect antibacterian, în funcție de tipul de solvent utilizat pentru extracție și, de asemenea, de tulpina luata in studiu, excepția a fost făcută de extractul cloroformic care nu a prezentat niciun efect antibacterian asupra tulpinilor selectate în studiu.

Keywords: collagen, minocycline, spongious delivery systems, freeze-drying

Introduction

Phytotherapy has become a resource in medicine, for its prevention purpose, and also for its use in the treatment of different affections. The use of plants and herbs for the purpose of cure has become attractive all over the world in the last decades [1].
Medicinal plants, and also spontaneous or crop plants are used as empirical therapy. Some active biological compounds from plants present antimicrobial effects; their action mechanisms sometimes assure a pathway to treat some infections determined by the antibiotic-resistant microorganisms [2]. An increased attention was focused on finding new "natural" sources due to the side effects caused by the administration of antibiotics as well as the resistance gained by many microbial strains.
Many plant species (spontaneous, cultured, medicinal) showed pharmaceutical and antimicrobial properties [3-12] therefore testing the possible antimicrobial effects of different parts of plant extracts is recommended.
Gnetum africanum is a wide use plant due to its nutritional value, high contents of proteins and minerals, thus it is used in food, animal feed and medical purposes for treating certain diseases. The antibacterial and antifungal properties of *G. africanum* extracts are based on the identified phytochemicals including tannins, flavonoids, terpenoids, alkaloids, saponins, and phenols [13].
The objectives of this study were to evaluate the phytochemical composition of *Gnetum africanum*

leaves extracts in methanol, chloroform and n-hexane, and to highlight their possible antimicrobial effects on different antibiotic-resistant microorganisms.

Materials and Methods

Plant collection and identification

G. africanum plants have been obtained from the market Eke-Awka (Anambra State, Southeastern Nigeria). *G. africanum* was brought from the origin country in the form of plant material. The leaves were removed from their stems and air dried under laboratory conditions, protected from light, for 14 days. After drying step, the plant material was milled into a fine powder. The obtained homogeneous mixture was further used for the extraction step.

Extraction procedure

In order to extract the desired chemical components from the plant material for further separation, the extraction of hydrophilic compounds uses polar solvents such as methanol (100 mL) and for the extraction of lipophilic compounds, solvents such as chloroform and hexane (100 mL) were used. The reaction was performed under magnetic stirring at room temperature for a period of 24 hours. The obtained extract was transferred to a clean tube and submitted to evaporation under nitrogen stream. The residue was filtrated using nylon membrane filter and reconstituted in 1 mL with the corresponding organic solvent for GC-MS analysis.

The extraction efficiency has been calculated based on the dried weight.

Efficiency (%) = (m_1/m_2) x 100, where m_1 represents the weight of extract obtained after vaporization of solvent, and m_2 represents the initial weight of fresh powder.

Tested concentrations

Methanol, chloroform and hexane extracts of *G. africanum* were divided in 3 experimental groups, each of them in 3 different concentrations of total phenol content determined according to Popescu R. *et al*. [14]: methanol extract of *G. africanum* (Group A) in concentrations of: 265.50 mg/mL (c1), 132.75 mg/mL (c2) and 66.37 mg/mL (c3); hexane extract of *G. africanum* (Group B) in concentrations of: 31.85 mg/mL (c1), 15.92 mg/mL (c2) and 7.96 mg/mL (c3); chloroform extract of *G. africanum* (Group C) in concentrations of: 44.25 mg/mL (c1), 22.10 mg/mL (c2) and 11.05 mg/mL (c3).

Derivatization procedure

Derivatization procedure was performed in order to increase the volatility and the thermal stability of analytes for chromatographic separation. Sylilation was the reaction of choice in this purpose. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was applied to every extract. The reaction was allowed to perform 7 hours in dark, at room temperature, and then the samples were submitted to GC-MS analysis.

Gas-chromatography mass spectrometry (GC-MS) analysis

GC-MS analysis of *Gnetum africanum* extract was achieved using a 450 GC – 240 MS (Varian, California USA). Separation of compounds was performed using a capillary column (VF-1ms), with 30 m long, an inner diameter of 0.25 μm and a film thickness of 0.25 μm. 1 μL of each extract was injected into the GC-MS using a micro syringe. Injection port temperature was set at 300ºC. Column temperature was set as follows: initial temperature was set at 60ºC maintained for 3 min, followed by 10ºC/min increase till 290ºC, with a stationary time of 6 min. Total run time was 30 min. Helium was used as carrier gas at a constant flow rate of 1.2 mL/min. Scanning was performed under 70 eV current emission, and fragments were monitored through 50 to 450 *m*/z. The ionized compounds were identified by comparing their spectra to those of the Wiley, PMW and NIST mass spectral libraries.

For effective comparison of the obtained chromatographic data, and in order to eliminate factors that can mask the chemical fingerprint of bio-active compounds, a scaling step was performed on each chromatographic intensity across all extracts. Based on chemical information regarding the identification of bio-active compounds and also based on the abundance of chromatographic peaks corresponding to the compounds in the extracts, a stock solution corresponding to each extract was prepared and it was successively diluted to obtain 3 concentration levels for each extract.

Antimicrobial susceptibility tests

Microorganisms testing

The antimicrobial activity was studied using Gram-positive bacterial strains (*Staphylococcus aureus, Bacillus cereus*), as well as Gram-negative bacterial strains (*Escherichia coli, Klebsiella spp., Enterobacter aerogenes*). The antibiotic-resistant microorganisms used in the present study were isolated in the Microbiology Laboratory of Emergency County Hospital "Pius Brinzeu" Timişoara, Romania, from patients.

Determination of Minimum Inhibitory Concentration (MIC) – Disc-diffusion method

Antimicrobial tests for the selected microorganisms were carried out using a Kirby-Bauer disc-diffusion susceptibility test [15-16]. A small amount of each microbial culture was diluted in sterile 0.9% sodium chloride solution until the turbidity was equivalent to McFarland standard no. 0.5. The suspensions were further diluted 1:10 in medium CHROM agar (Oxoid) and then spread on sterile Petri plates. Blank sterile antimicrobial susceptibility discs were applied on the agar surface in Petri plates. Afterwards, 10 μL of each sample was added on

disc surfaces. Commercially available antimicrobial susceptibility test discs were used as positive controls for antibiograms. The plates were incubated at 37°C for 24 h. After incubation, inhibitory areas around the discs were measured.

Cellular viability test

100 µL of culture in Mueller Hinton broth with a turbidity equivalent to McFarland standard no. 0.5 was transferred to a 96-well plate. Following that, 50 µL of the extract was added. Samples were incubated at 37°C for 6 hours. 10 µL of 0.5% tri-phenyltrazolium chloride (TTC) 2,3,5-adduct were added and the samples were incubated at 37°C for another 2 hours. The samples analysis was performed at 460 nm with the TecamSunrise spectrophotometer. The rate of inhibition was determined using the following formula:

Rate of inhibition:

$$(\%) = [(Ac - Ap)/(Ac)] \times 100,$$

where *Ac* represents the absorbance of the control solution, and *Ap*, the absorbance of the sample. All experiments were performed in triplicate, the presented values are expressed as average and standard errors. One-way ANOVA followed by Bonferroni's post-tests were used to determine the statistical difference between the effects of extracts *vs*. gentamicin (Gn) and sulfamethoxazole -trimethoprim (SXT) used as control: Gn for *S. aureus* and *B. cereus* and SXT for *E. coli*, *Klebsiella spp.*, and *Enterobacter aerogenes*. *p < 0.05, **p < 0.01 and ***p < 0.001

Results and Discussion

Chromatographic analysis

Tables I, II and III present the volatile compounds obtained by extraction of *Gnetum africanum* with different solvents based on gas chromatography-mass spectrometry (GC-MS), using a 450-GC coupled with 240-ion trap MS.

Table I

Identified compounds in the derivatized chloroformic extract of *Gnetum africanum*

Retention time [min]	Compound	Area [%]
4.822	trifluoromethyl-bis-(trimethylsilyl)-methyl ketone	3.39
5.107	Pentalin	1.99
7.128	laevulic acid trimethylsilyl ether	1.95
7.637	glucose 5-trimethylsilyl	0.97
8.663	gluconic acid ɣ-lactone, 5methoxymine, tri(trimethylsilyl)	2.11
9.530	glycerol, tris(trimethylsilyl ether)	4.61
9.821	succinic acid trimethylsilyl ester	1.33
10.477	nonanoic acid trimethylsilyl ester	1.97
13.998	lauric acid trimethylsilyl ether	1.55
14.450	suberic acid trimethylsilyl ester	1.76
15.499	azelaic acid, bis-trimethylsilyl ester	4.66
16.007	hexahydrofarnesyl acetone	3.40
16.092	myristic acid trimethylsilyl ester	3.08
17.057	n-pentadecanoic acid trimethylsilyl ester	1.97
17.983	**palmitic acid trimethylsilyl ester**	**16.04**
18.628	cis-10-heptadecanoic acid trimethylsilyl ester	1.78
18.866	**heptadecanoic acid trimethylsilyl ester**	**7.69**
19.205	phytol, trimethylsilyl ether	2.30
19.494	trimethylsilyl 9E-9-octadecanat	11.52
19.736	**stearic acid trimethylsilyl ester**	**19.11**
20.060	cis-11-eicosenoic acid trimethylsilyl ester	1.86
20.427	cis-10-nonadecenoic acid trimethylsilyl ester	4.95

Table II

Identified compounds in the derivatized hexane extract of *Gnetum africanum*

Retention time [min]	Compound	Area [%]
4.828	**trifluoromethyl-bis-(trimethylsilyl)-methyl ketone**	**14.70**
7.126	laevuric acid trimethylsilyl ester	1.04
8.661	gluconic acid, ɣ-lactone-5-methoximine, tri(trimethylsilyl)	2.19
8.732	**2-phenylindolizine**	**11.74**
9.184	octanoic acid trimethylsilyl ester	0.52
9.812	succinic acid trhymethylsilyl ester	0.65
10.474	nonanoicaicdtrimethylsilyl ester	1.77
11.712	decanoic acid trimethylsilyl ester	0.45
13.997	dodecanoic acid trimethylsilyl ester	1.48
14.441	suberic acid trimethylsilyl ester	0.95
15.059	tridecanoic acid trimethylsilyl ester	0.73

Retention time [min]	Compound	Area [%]
15.273	tetradecyltrimethylsilyl ether	0.97
15.412	n-pentanoic acid trimethylsilyl ester	1.02
16.489	palmitic acid trimethylsilyl ester	2.90
17.049	**n-pentadecanoic acid trimethylsilyl ester**	**5.35**
17.405	**heptadecanoic acid trimethylsilyl ester**	**5.08**
17.655	5-chloro-6-nitrocholestane-3-one	0.84
19.501	cis-11-eicosenoic acid Trimethylsilyl ester	3.31
20.058	(E)-3,7,11,15-tetramethylhexadec-2-enoic acid trimethylsilyl ester	6.55
20.427	**cis-10-nonadecenoic acid trimethylsilyl ester**	**17.00**
21.350	**arachidic acid trimethylsilyl ester**	**6.39**
22.575	1-monopalmitin trimethylsilyl ether	4.00
22.898	docosanoic acid trimethylsilyl ester	4.06
24.428	lanost-8-3,7-dione	6.33

Table III

Identified compounds in the derivatized methanolic extract of *Gnetum africanum*

Retention time [min]	Compound	Area [%]
4.732	1,2-dimethylpirolydine	7.35
5.191	3-HO-5-N-pyrolydinomethyl-isoxazole	4.61
6.413	**piperidine, 1-(2-methylpropenyl)**	**4.92**
6.567	**2,5-bis-(1,1,3,3-tetramethylbutyl) thiophene**	**13.09**
6.774	**piperidine, 3-dimethylamino-1-methyl**	**5.51**
8.650	2,5-dimethyl-4-benzyl-pyridine	15.17
10.170	9,9-dimethyl-3,7-diazobicyclo[3.3.1]nanone	7.56
11.865	**5,7-dimethyl-1,3-diazaadamantan-6-one hydrazone**	**20.84**
12.814	**1,8-dimethyl-3,6-diazahomoadamantan-9-spiro-2'-oxirane**	**20.95**

Microbiological analyses

The bacterial strains used in the present study showed resistance to several antibiotics; the results of antibiograms are presented in Table IV.

Table IV

Sensibility and resistance to antibiotics of tested bacterial strains

Bacteria / Sensibility	*S. aureus*	*B. cereus*	*E. coli*	*Klebsiella spp.*	*Enterobacter aerogenes*
Sensitive	Fox, E, Da, Gn, Cip, Sxt, Tec, Lzd	Cro, Sam, Caz, Tob, Fep, Imi, Ak, Gn, Cip, Lev	Imi, Ak, Net	Sxt, Cs	Tzp, Caz, Sam, Sxt, Mem, Imi, Ak, Lev
Intermediary		Sxt	Tzp, Sam, Gn, Sxt	Lev, Ak	
Resistant	P		Cxm, Fep, Cro, Caz, Cip,	Mem, Imi, Gn, Cip, Cxm, Fep, Tzp, Cz, Caz, Sam	Cxm, Cz, Pip, Cip, Gn

Fox - Cefoxitin, E - Erythromycin, Da - Clindamycin, Gn - Gentamicin, Cip - Ciprofloxacin, Sxt – Trimethoprim-Sulfamethoxazole, Tec - Teicoplanin, Lzd - Linezolid, Cro - Ceftriaxone, Sam - Ampicillin + Sulbactam, Caz - Ceftazidime, Tob - Tobramycin, Fep - Cefepime, Imi - Imipenem, Ak - Amikacin, Lev - Levofloxacin, P - Penicillin, Net - Netilmicin, Tzp - Piperacillin + Tazobactam, Cxm - Cefuroxime, Cs - Colistin, Mem - Meropenem, Cz - Cefazolin, Pip - Piperacillin

The inhibition rate values determined for each bacterial strain and for each type of extract at each concentration were centralized and plotted.

The values of the inhibition zones were obtained by comparatively testing of the extracts *vs*. gentamicin (Gn) and, respectively sulfamethoxazole-trimethoprim (SXT) which were used as references. The diameters of the inhibition zones differ depending on the species: for Gentamicin - *S. aureus* 15 mm and *B. cereus* 15 mm, respectively for the Trimethoprim/Sulfamethoxazole - *E. coli*, *Klebsiella spp* and *Enterobacter aerogenes* 17 mm.

In the case of the *G. africanum* methanolic extract the inhibition rates ranged between 14.5 mm and 7 mm. In bacterial strains *S. aureus* and *E. aerogenes* at the concentration of c1 in the methanolic extract, inhibition values of 14.2 and 14.5 mm were recorded, indicating an intermediate susceptibility to the action of the extract according to Intorasoot A. *et al.* [17]. At the c2 concentration of the methanolic extract on *S. aureus*, *B. cereus*, *E. coli*, *Klebsiella spp* and *E. aerogenes* strains, the inhibition rates also indicated an intermediate sensitivity (compared to the values recorded for Gn and Sxt) (Figure 1a). Hexane extract

of *G. africanum* presented an intermediate antibacterial action on the *B. cereus* strain at all 3 concentrations tested, with inhibition rates ranging from 14.5 to 11.2 mm. On the *S. aureus* strain, the hexane extract showed intermediate antibacterial action only at using the c1 concentration (Figure 1b). The antibacterial effect of hexane extract of *G. africanum* on the other types of bacterial strains hasn't been notified, the values of the inhibition ranges being of 7 mm or absent.

The chloroform extract of *G. africanum* at the tested concentrations, didn't manifest any antibacterial effect, the values of inhibition ranges being absent or maximum at 10.8 mm (Figure 1c).

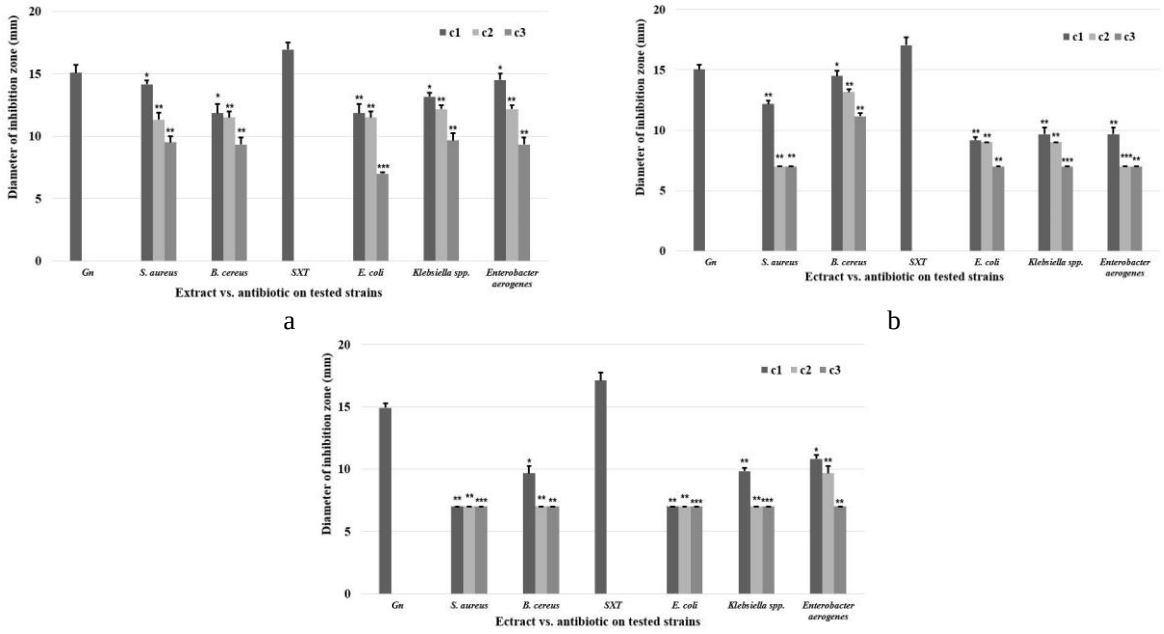

Figure 1.

Comparative inhibition of *G. africanum* extracts *vs*. Gn and SXT on the bacterial strains (a - methanolic extract; b - n-hexane extract; c - chloroformic extract) (*p < 0.05, **p < 0.01 and ***p < 0.001)

The use of the cellular viability test to the isolated bacterial strains, under the action of the *G. africanum* extracts, allowed the determination of the inhibition range and implicitly of the presence/absence of the antibacterial effects of these extracts. Against *S. aureus*, the extract of *G. africanum* in hexane, based on the value of the inhibition range, exhibits an intermediary antibacterial effect, at the use of concentrations c1 and c2. Intermediary sensibility presents also *S. aureus* at the application of the extract of *G. africanum* in methanol only at c1. The extract of *G. africanum* in chloroform doesn't exhibit an antibacterial effect on the *S. aureus* strain, the values of the inhibition range being under 50% (Figure 2a).

The inhibition values of the hexane-based extract are between 50 - 80%; the concentration c1 can be considered as sensitive, while c2 and c3 exhibit an intermediary sensibility. The concentrations c1 and c2 of the chloroformic extractpresent intermediary sensibility (Figure 2b). The methanolic extract of *G. africanum* doesn't present an antibacterial effect on *B. cereus*, the inhibition values being below 50%.

It was observed that *E. coli* exhibits an intermediary sensibility in the case of c1 and c2 concentrations of methanolic extract, respectively in the case of concentration c1 of the extract in hexane. The chloroformic extract of *G. africanum*, at the tested concentrations, doesn't present any antibacterial effect (Figure 2c).

The inhibition values are below 50%, with the exception of the methanolic extract at c1 concentration for *Klebsiella spp*. Consequently, an intermediary sensibility is manifested by the *Klebsiella spp*. strain only in case of the c1 methanolic extract (Figure 2d).

The values of the determined inhibition ranges at the application of the 3 types of extracts are under the level of 50%, in order to be considered as manifesting an antibacterial effect, excepting the methanolic extract of c1, where the bacterial strain *E. aerogenes* manifests an intermediary sensibility (Figure 2e).

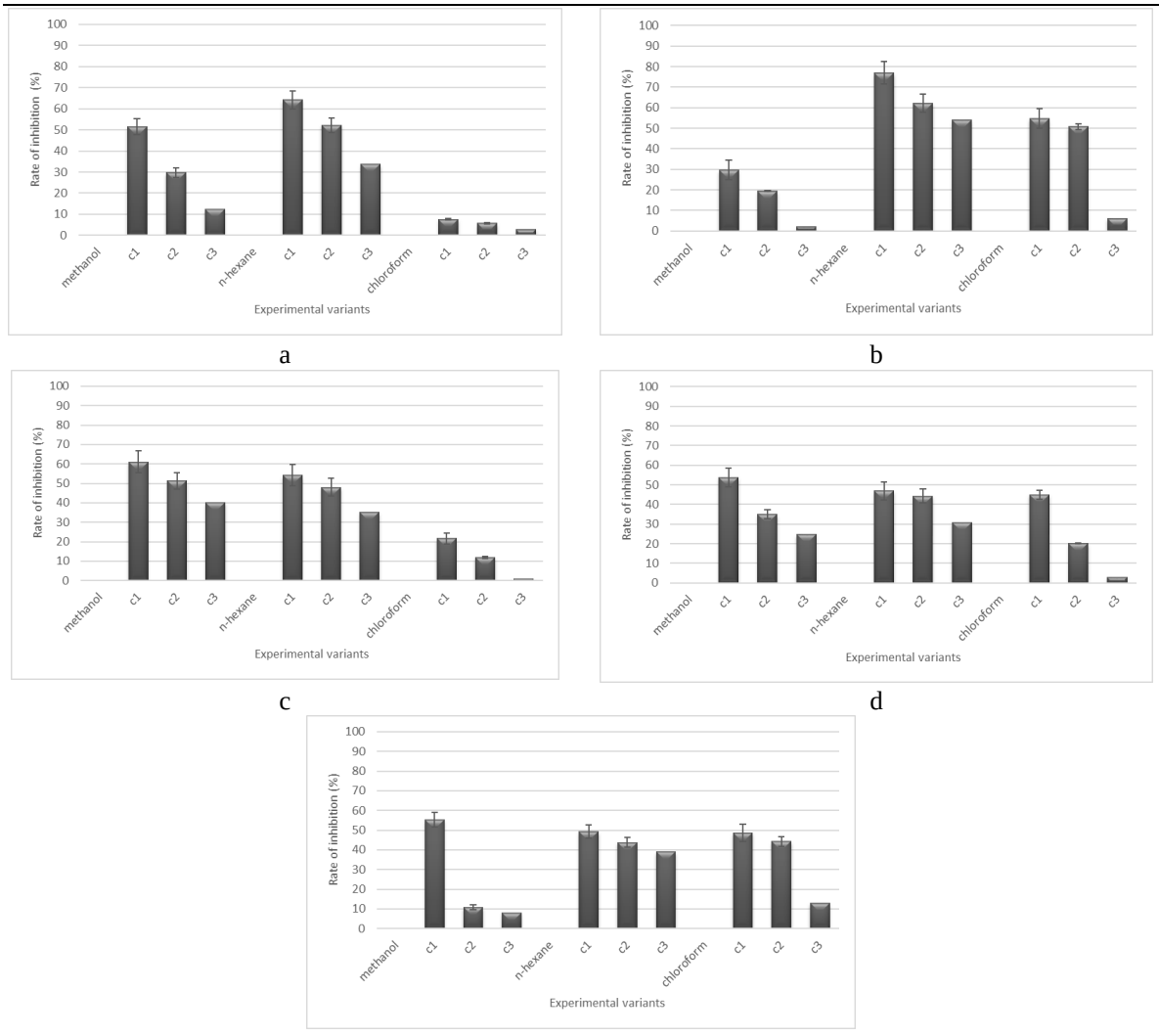

Figure 2.
Inhibition range (%) of the bacterial strains at the action of *G. Africanum* extracts (a- *S. aureus*, b- *B. cereus*, c- *E. coli*, d- *Klebsiella spp.*, e- *E. aerogenes*)

Previous studies have mentioned the antibacterial effect of aqueous and methanol extracts from *Gnetum africanum* leaves on standard microbial strains (*Escherichia coli* (ATCC25922), *Staphylococcus aureus* (ATCC25923) and *Candida albicans* (ATCC 10231). The results have indicated the fact that both extracts had inhibitor effect dependent on the dose on the increase of *S. aureus*, with maximum inhibition areas of 13.30 and 13.10 mm at 200 mg/mL for the aqueous respectively ethanolic extracts. Although, the extracts didn't present antibacterial activity on the *E. coli* strain which might suggest the inefficiency against Gram-bacteria [7]. Comparing the results obtained by us with the ones presented in the previous study [7], we ascertain the fact that the determined values at the use of our extracts (methanolic, n-hexane and chloroform) are much lower, the possible explanations being the following: less concentrated extracts, the antibiotic-resistant microorganisms, the type of solvent used for extraction.

The observed antimicrobial activity can represent a consequence of the rich phytochemistry of leaves, due to the fact that the preliminary phytochemical screening indicated the fact that leaves contain alkaloid, tannin, saponin, sterol, flavonoid, terpenoid, glycoside cyanogen and antraquinone [7]. The anti-bacterial and antifungal effects of flavonoids and tannins [18], terpenoids (acting on the integrity of cellular membranes and manifesting an inhibitor action on microorganisms) [19], saponins (a special category of glycosides used due to the large range of pharmacology and medicinal features) [20], and of sterols and phenols (which induce a mutagenicity at the level of the cellular DNA) are well known.

Applying the cell viability test to the bacterial strains isolated under the action of *G. africanum* extracts allowed the determination of inhibition rate, and, implicitly, the presence or absence of the antibacterial effect on the tested extracts. On *S. aureus* bacterial strain, the hexane extract of *G. africanum* based on

the inhibitory rate value, showed an intermediate antibacterial effect at the application of c1 and c2 concentrations. An intermediate sensitivity on *S. aureus* was shown only at c1 concentration of methanolic extract.

During the mass analysis, there were identified saturated and unsaturated fatty acids, adamantan derivatives like 1,8-dimethyl-3,6-diazahomoadamantan-9-spiro-2'-oxirane and 5,7-dimethyl-1,3-diazaadamantan-6-one hydrazine, piperidine derivatives like 1-(2-methylpropenyl) piperidine and, 3-dimethylamino-1-methyl piperidine, steroids like 3,5-stigmastadien-7-one, all of them presenting important biological activity as mentioned above.

Conclusions

The results showed that different compounds were identified by applying different polarity solvents during the extraction step. The presence of various bio-active compounds was proven by GC-MS analysis. Based on our results, it could be concluded that *G. africanum* contains various bio-active compounds and it is recommended as a plant with a phyto-pharmaceutical importance.

The extracts of *Gnetum africanum* showed different antibacterial effects, depending on the type of solvent and the tested concentration. Based on the inhibition values, the methanolic extract of *G. africanum* determined a decrease of sensibility as follows: *Staphylococcus aureus* > *Enterobacter aerogenes* > *Bacillus cereus* = *Klebsiella spp.* > *Escherichia coli*. Hexane-based extract of *G. africanum* at the tested concentrations, determined a decrease of sensibility of the tested strains, as follows: *Bacillus cereus* > *Staphylococcus aureus* > *Enterobacter aerogenes* = *Escherichia coli* > *Klebsiella spp*. The chloroformic extract of *G. africanum* at the tested concentrations didn't exhibit any antibacterial effect on the strains selected in the study.

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