European Journal of Chemistry

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Influence of the physicochemical parameters of solvents in the extraction of bioactive compounds from *Parinari macrophylla* Sabine (*Chrysobalanaceae*)

Mamadou Baldé [1,2], Saïd Ennahar [1], Stéphanie Dal [3], Séverine Sigrist [3], Alassane Wélé [2], Eric Marchioni [1] and Diane Julien-David [1,*]

[1] *Laboratory of Analytical Chemistry of Bio-Active Molecules and Pharmacognosy, Hubert Curien Multidisciplinary Institute, UMR 7178, University of Strasbourg, CNRS, 74, Route du Rhin, 67400 Illkirch-Graffenstaden, France*
mamadou.balde@etu.unistra.fr (M.B.), ennahar@unistra.fr (S.E.), eric.marchioni@unistra.fr (E.M.), diane.julien-david@unistra.fr (D.J.D.)
[2] *Laboratory of Physical Chemistry, Mineral Chemistry, Organic Chemistry and Therapeutic Chemistry, Faculty of Medicine, Pharmacy and Odontology, Cheikh Anta Diop University (UCAD), BP 5005, Dakar, Senegal*
alassane.wele@ucad.edu.sn (A.W.)
[3] *UMR DIATHEC, EA 7294, Federation of Translational Medicine of Strasbourg (FMTS), University of Strasbourg, Rene Leriche Boulevard, 67200 Strasbourg, France*
dalstephaniepro67@gmail.com (S.D.), s.sigrist@defymed.com (S.S.)

* *Corresponding author at: Laboratory of Analytical Chemistry of Bio-Active Molecules and Pharmacognosy, Hubert Curien Multidisciplinary Institute, UMR 7178, University of Strasbourg, CNRS, 74, Route du Rhin, 67400 Illkirch-Graffenstaden, France.*
Tel: +33.3.68854326 Fax: +33.3.68854325 e-mail: diane.julien-david@unistra.fr (D. Julien-David).

RESEARCH ARTICLE

10.5155/eurjchem.9.3.161-167.1723

Received: 23 April 2018
Received in revised form: 31 May 2018
Accepted: 02 June 2018
Published online: 30 September 2018
Printed: 30 September 2018

KEYWORDS

TEAC
ORAC
Plants
Extraction
Cell viability
Solvent effects

ABSTRACT

The extraction of bioactive compounds from medicinal plants requires methods which are as diverse as the chemical nature of the compounds themselves. In this study, a 96-well microplate was used where solvent mixtures spanning wide ranges of selectivity and polarity were tested with the objective of extracting a broad range bioactive compounds from plant material. Microplate wells were filled with plant material and the solvents and their mixtures were added. The obtained extracts were assessed in terms of their total antioxidant activity, oxygen radical absorbance capacity and effects on cell viability. An aqueous extract, generally used by traditional therapists, was also included in the study. The results showed that the extracts using methanol with acetic acid (0.1%, *v*:*v*), chloroform/ethanol, butanol/DMF, butanol/acetonitrile, ethylene glycol with acetic acid (0.1%, *v*:*v*), MTBE/DMSO, ethylene glycol, pentane/ethanol (*v*:*v*), ethanol, DMF, DMF with acetic acid (0.1%, *v*:*v*), DMSO, DMSO with acetic acid (0.1%, *v*:*v*) and THF had a higher antioxidant activity than the aqueous extract. Extracts with greater antioxidant activity than the aqueous extract were obtained largely from solvent mixtures with the exception of ethanol, DMF, DMSO and THF. The antioxidant activity obtained in TEAC varied between 1474.1±4.4 and 3183.0±16.0 µmol TE/g dry extract respectively for aqueous and THF extracts; in ORAC between 1727.7±8.4 and 2683.5±11.7 µmol TE/g dry extract for aqueous and DMSO acetic acid 1%, respectively, with mean ±SEM. In TEAC the THF extract had the highest antioxidant potential with 3183.0±16.0 µmol TE / g dry extract. The DMSO acetic acid (0.1%, *v*:*v*) extract had the highest antioxidant potential in ORAC with 2683.5±11.7 µmol TE / g dry extract. Cell viability test using β-pancreatic cells showed that only the acidified methanol extract was toxic after one hour of incubation. After 24 hours, cell viability was less than 70% for extracts using butanol/acetonitrile, MTBE/DMF, acidified methanol, pentane/ethanol and acidified DMF.

Cite this: *Eur. J. Chem.* **2018**, *9(3)*, 161-167 **Journal website**: www.eurjchem.com

1. Introduction

The bioactive compounds present in natural plants belong to several chemical groups and are of very diverse natures [1]. Their extraction requires solvents where they can dissolve and then be recovered by physicochemical techniques such as filtration, evaporation, precipitation, etc. Choosing the right extraction solvent is therefore fundamental in order to extract the compounds of interest selectively and in high amounts. This in turn has a direct impact on the outcome of the biological activity assays [2-4].

Most studies use the conventional approach of targeting a chemical group of compounds by using solvents where they are likely to dissolve [5]. Often, a single solvent or solvent mixture is used based on published data without taking into account the specificities of the biological matrices and the diversity of the bioactive compounds they contain. Polar compounds such as polyphenols are extracted by polar solvents or mixtures of polar solvents [6]. This may lead to less efficient extractions in terms of selectivity and yield or even to missing specific molecules. Testing various solvent combinations with arrays of intermediate polarities and selectivities

http://dx.doi.org/10.5155/eurjchem.9.3.161-167.1723

would allow a more guided choice of extraction conditions with regard to the targeted bioactive molecules.

In the present study, a new system was set up which consists of a 96 well microplate in which several solvents and their mixtures were used to extract a diversity of bioactive compounds. The solvents were selected according to their physicochemical parameters (selectivity, polarity, boiling point, pH, viscosity, dipole moment, *etc.*) [7-11].

To evaluate the biological activity of the extracts, several methods can be used, including antioxidant, antiparasitic and cytotoxic tests [12-15]. In this study, the extraction efficiency of the different solvents is evaluated by the determination of antioxidant and cytotoxic activities. Anti-oxidant activities were measured using physico-chemical tests: Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) and the toxicity of the extracts was evaluated using biological tests on RiNm5F cells (cell viability test). This extraction solvent selection system was tested on a Senegalese plant, *Parinari macrophylla*, used by traditional therapists for its anti-diabetic properties. The results obtained were compared with those of the aqueous extract, a solvent conventionally used.

2. Experimental

2.1. Solvents used for extraction

All solvents used in this study were of analytical grade. Ethylene glycol, benzyl alcohol, ethanol, 4-methyl-2-pentanone, dichloromethane, ammonium hydroxide, acetic acid and isooctane were purchased from Sigma Aldrich (St Louis, USA).

Dimethylformamide (DMF) and dimethyl sulfoxide (DMSO) were purchased from Prolabo (France). Acetonitrile, chloroform, cyclohexane were purchased from Fischer (UK). Tetrahydrofuran (THF), methanol, *n*-heptane, pentane, hexadecane, ethyl acetate were purchased from VWR (France). Isopropanol and n-butanol were purchased from Carlo Erba (France). Heptanol, methyl *tert*-butyl ether (MTBE), *n*-octanol, 4-methyl-2-pentanol, and xylene were purchased from Merck (Germany). Diethyl ether, petroleum ether were purchased from SdS (France). Toluene, methyl ethyl ketone (MEK) were purchased from Fluka (France) and triethylamine was purchased from ACROS (Belgique).

2.2. Reagents for physical and chemical tests

2,2′-Azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Trolox: (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, potassium persulfate, 2,2′-azo-*bis*(2-methylpropionamidine) dihydrochloride (AAPH), and fluorescein were purchased from Sigma Aldrich (Steinheim, Germany).

The phosphate buffer solution used was prepared as follows (PBS: 137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 10 mM di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$), 1.76 mM potassium phosphate (KH_2PO_4) dissolved in 1 L of Milli-Q water.

2.3. Material and reagents for biological tests

The Roswell Park Memorial Institute Medium (RPMI-1640) and fetal calf serum were purchased from Sigma (St Louis, USA), the Cell Titer-96 aqueous solution from Promega and Trypan Blue Dye, 0.4% from BIO-RAD. Trypsin-EDTA (1X) 0.05%, PBS pH = 7.2 (1X) and the antibiotic-antimycotic solution were purchased from Gibco Invitrogen (Grand Island, USA). The cells used for the toxicity tests were beta cells of a rat insulinoma tumor line. They were obtained from the American Type Culture Collection (ATCC, Manassas, USA).

2.4. Plant material

The leaves of *Parinari macrophylla* were harvested manually in Pakour, in the Kolda region of southern Senegal. They were dried, reduced to powder in an AGREX hammer mill, packed in dark plastic bags and kept at room temperature.

2.5. Extraction procedure

Microplate wells were filled with plant material (leaves powder) and excess was removed by shaving. The average mass per well was 25±0.3 mg. Then, the contents were transferred into a deep well microplate consisting of 96 2-mL wells (ThermoFisher Scientific, Illkirch, France). Solvents and their mixtures (1.5 mL) were then added according to the layout shown in Table 1, followed by the addition of 1.5 mL of each solvent or solvent mixture. Extraction was performed by maceration and the microplates were subjected to automatic stirring at 150 rpm for 48 hours (Bioblock Scientific Shaker, 74402). Then, vacuum filtration was carried out using an AcroPrepTM 96 Filter microplate with 40 µm pore size (Pall, Saint-Germain-en-Laye, France). Then, dry evaporation was performed at SpeedVac SDP121P concentrator (Thermo Scientific, Waltham, Massachusetts, USA) and whose enclosure was heated to 40 °C to remove all solvents. Since wells contained solvents with various boiling points, water was gradually added to wells with easily evaporable solvents to avoid drying and caramelization. Finally, the extracts were redissolved in 1 mL of DMSO, which constituted the stock solutions for chemical tests. For biological tests, the extracts were dissolved in RPMI-1640 medium.

2.6. Evaluation of the antioxidant activity

2.6.1. Trolox equivalent antioxidant capacity (TEAC)

This method is based on electron transfer and uses $ABTS^{\cdot+}$, a chromophore radical which is a blue-green cation formed when ABTS reacts with potassium persulfate [16,17]. $ABTS^{\cdot+}$ has absorption maxima at wavelengths of 412, 645, 734 and 815 nm [18]. In the presence of antioxidant compounds, $ABTS^{\cdot+}$ free radical is captured, which leads to a loss of color and therefore a reduction in the measured absorbance quantitatively linked to the concentration of antioxidants [19-22]. Trolox was used as a reference for quantitative assessment and calibration was done using a range of concentration in Milli-Q water (20, 80, 200, 500 and 900 µmol/L). DMSO, used to redissolve extracts, served as a negative control. Prior to testing, extracts' stock solutions were diluted in Milli-Q water with 3% DMSO (*v*:*v*). Ten µL of each diluted extract were then deposited in a microplate well followed by 200 µL of $ABTS^{\cdot+}$ at 7 mmol/L in PBS. After 10 minute incubation at 37 °C, absorbance at 734 nm was read in a Vario Skan spectrophotometer (ThermoFisher scientific). Experiment were carried out in triplicate.

2.6.2. Oxygen radical absorbance capacity (ORAC)

The ORAC test relies on the oxidation of a fluorescent probe (fluorescein) by free radicals, which are often peroxylic radicals, but may also be hydroxyl radicals. These free radicals are produced by a radical generator (AAPH) [23,24]. During the experiment, the free radicals damage the probe and thus reduce the intensity of the fluorescence.

Table 1. Microplate layout used for multiple-solvent extraction.

	1	2	3	4	5	6
A	Water	Water / Acetic acid 1% *v*:*v*	Water / NH_4OH 0.05 M	Methanol	Heptanol / DMSO	Heptanol / Methanol
B	DMSO	DMSO / Acetic acid 1% *v*:*v*	DMSO / NH_4OH 0.05M	Methanol / Acetic acid 1% *v*:*v*	MTBE / DMSO	MTBE / Methanol
C	Ethylene glycol	Ethylene glycol / Acetic acid 1% *v*:*v*	Ethylene glycol / NH_4OH 0.05 M	Methanol / NH_4OH 0.05 M	Ethyl acetate / DMSO	Ethyl acetate / Methanol
D	DMF	DMF / Acetic acid 1% *v*:*v*	DMF / NH4OH 0.05 M	Isopropanol	MEK / DMSO	MEK / Methanol
E	Acetonitrile	Acetonitrile / Acetic acid 1% *v*:*v*	Acetonitrile / NH_4OH 0.05 M	Isopropanol / Acetic acid 1% *v*:*v*	Dichloromethane / DMSO	Dichloromethane / Methanol
F	THF	THF / Acetic acid 1% *v*:*v*	THF / NH_4OH 0.05 M	Isopropanol / NH_4OH 0.05 M	Toluene / DMSO	Toluene / Methanol
G	Benzyl alcohol	Benzyl alcohol / Acetic acid 1% *v*:*v*	Benzyl alcohol / NH_4OH 0.05 M	Butanol / Acetonitrile	Butanol / DMSO	Butanol / Methanol
H	Ethanol	Ethanol / Acetic acid 1% *v*:*v*	Ethanol / NH_4OH 0.05 M	Chloroform / Acetonitrile	Chloroform / DMSO	Chloroform / Methanol
	7	**8**	**9**	**10**	**11**	**12**
A	Heptanol / DMF	Heptanol	*n*-Octanol	Diethyl ether	Triethylamine	Petroleum ether
B	MTBE / DMF	MTBE	2-Methyl propanol	2-Methyl pentanol	Methyl-1-butanol	Methyl pentanone
C	Ethyl acetate / DMF	Ethyl acetate	Xylene / THF	Xylene / Ethanol	Xylene / Isopropanol	Xylene
D	MEK / DMF	MEK	Cyclohaxane / THF	Cyclohexane / Ethanol	Cyclohexane / Isopropanol	Cyclohexane
E	Dichloromethane / DMF	Dichloromethane	Isooctanol / THF	Isooctanol / Ethanol	Isooctanol / Isopropanol	Isooctane
F	Toluene / DMF	Toluene	Heptane / THF	Heptane / Ethanol	Heptane / Isopropanol	Heptane
G	Butanol / DMF	n-Butanol	Pentane / THF	Pentane / Ethanol	Pentane / Isopropanol	Pentane
H	Chloroform / DMF	Chloroform	Chloroform / THF	Chloroform / Ethanol	Chloroform / Isopropanol	Hexadecane

The degree of change in intensity reflects the amount of damage caused by free radicals. The addition of an antioxidant makes it possible to absorb the free radicals, which reduces the damage received by the probe and prolongs its fluorescence. To quantify the protection conferred by an antioxidant, a measurement of the area under the curve of the sample was made and compared to the area under the curve [25] of trolox as a reference antioxidant. A calibration curve was constructed using different concentrations of trolox in Milli-Q Water (20, 80, 200; 500 and 900 µmol/L). Prior to testing, extracts' stock solutions were diluted in Milli-Q water with 3% DMSO (*v*:*v*). Ten µL of each diluted extract were then deposited in a microplate well followed by 150 µL of fluorescein 8.5×10^{-8} mol/L in Milli-Q water. After a 10-minute incubation at 37 °C, AAPH at 153×10^{-3} mol/L in PBS [26-28] was automatic distributed in the microplate wells. The fluorescence kinetics was then monitored every 5 min for 120 min using a Vario Skan spectrophotometer with excitation and emission wavelengths of 485 nm and 530 nm, respectively. Experiment were carried out in triplicate. TEAC and ORAC results were expressed in µmol of equivalent trolox per gram of dry extract.

2.7. Cell viability test

The effect of extracts was evaluated on insulin secreting cell lines according to the method described by Auberval and slightly modified by Belhadj *et al.* [29,30]. Briefly, a rat insulinoma cell line (RINm5f beta) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells from passages 30-43 were used. The cells were grown in a controlled atmosphere at 37 °C under a 5% CO_2 in Roswell Park Memorial Institute (RPMI-1640, Sigma, St Louis, Etats-Unis) medium supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St Louis, MO, USA) and 1% antibiotic-antimycotic (ABAM; Gibco-Invitrogen, Grand Island, NY, USA). RINm5f beta cells were trypsinized using 0.05% trypsin ethylene diamine tetraacetic acid (EDTA; Sigma-Aldrich) at 80% confluence and loaded in a 96-well plate (Dutscher, Issy-les-Moulineaux, France) at a concentration of 3000 cells per well. The medium was changed every two days. Then, cells were incubated for 24 hours in serum-free medium. The cells were then incubated with the extracts for 1 hour, 24 hours at 37 °C and 5% CO_2. Viability of RINm5f beta cells was determined by measuring the mitochondrial activity with the Cell Titer 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). After treatment, 20 µL of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) and 100 µL of culture medium was added. Cells were incubated for 2 h at 37 °C in 5% CO_2, and the absorbance was measured at 490 nm using the microplate reader (iMarkTM, Biorad Laboratories Inc, USA). The color development was proportional to the number of viable cells. The results are presented in percentage (%) viability with respect to the negative control (cells treated in the same condition but only with culture medium). The toxicity data of the selected organic extracts were averaged ± SEM with three replicates (n = 3), and each replicate had six well repetitions. The data were then analyzed by One way-ANOVA analysis ($p < 0.05$) with Graph Pad Prism, version 6.01.

3. Results

The aqueous extract was considered as a reference for the selection of other extracts. Indeed, it is water that is generally used by traditional healers.

3.1. TEAC and ORAC results

The TEAC and ORAC results of the 96 extracts were sorted according to their antioxidant activity and divided into three groups (Figures 1-3) for a more convenient graphical representation.

In this study, for the fifteen extracts that were more active, the free radical scavenging potential was in the following order in TEAC method: water < chloroform/ethanol < butanol/DMF < ethylene glycol acetic acid 1 % (*v*:*v*) < butanol/acetonitrile < MTBE/DMF < methanol acetic acid 1 %

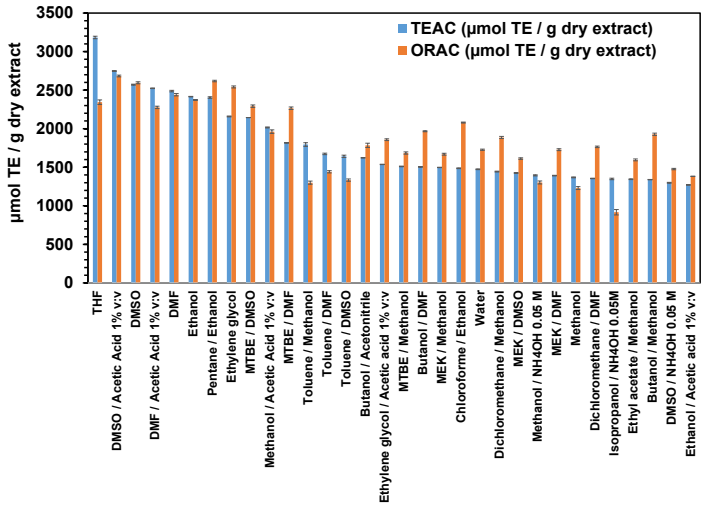

Figure 1. TEAC and ORAC results for extracts with high antioxidant activity.

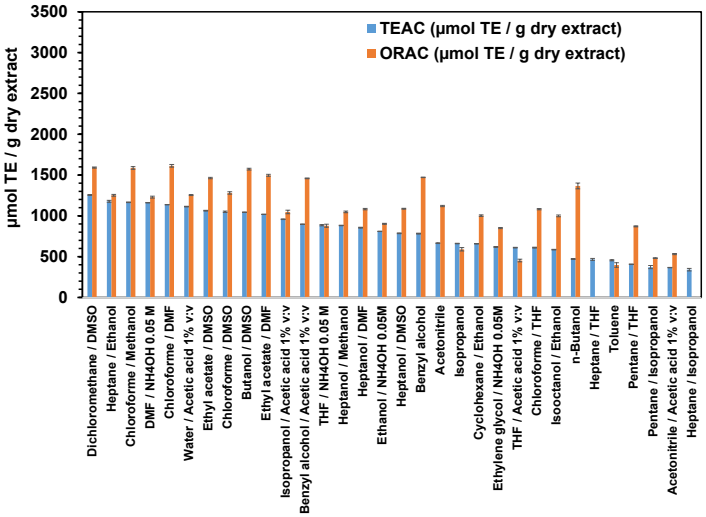

Figure 2. TEAC and ORAC results for extracts with moderate antioxidant activity.

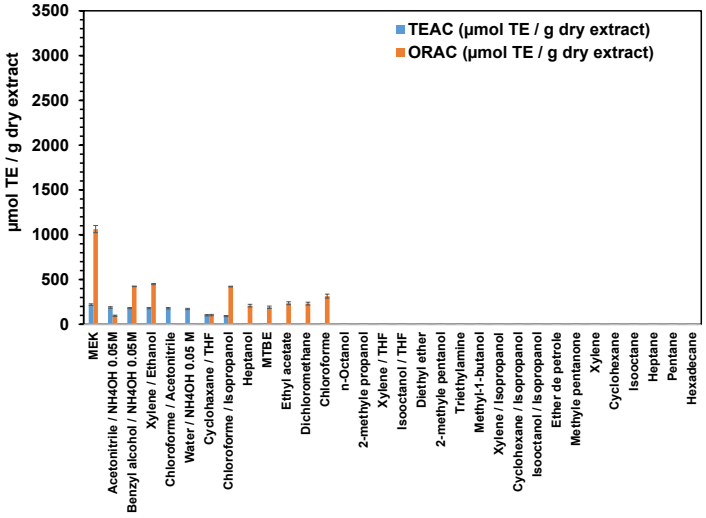

Figure 3. TEAC and ORAC results for extracts with low or no antioxidant activity.

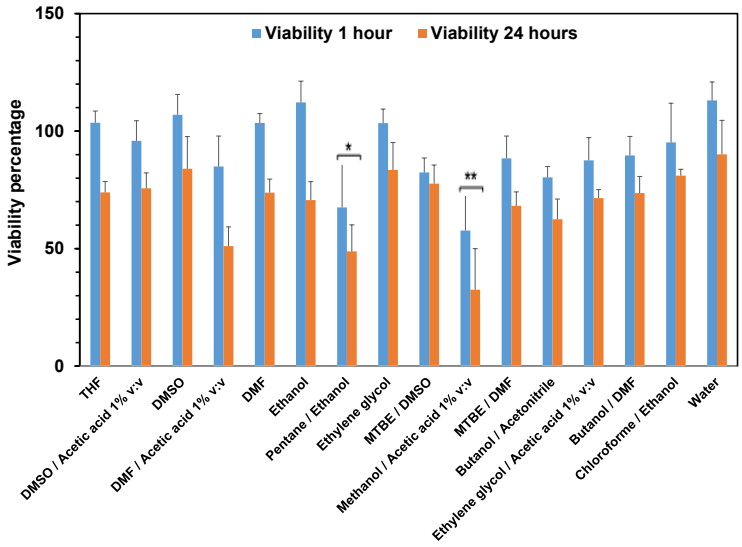

Figure 4. Viability percentage at 1 hour and 24 hours.

(*v*:*v*) < MTBE/DMSO < ethylene glycol < pentane/ethanol < ethanol < DMF < DMF 1% acetic acid < DMSO < DMSO acetic acid 1 % (*v*:*v*) < THF. In ORAC method free radical potential scavenging potential order was: Water < butanol/acetonitrile < ethylene glycol acetic acid 1 % (*v*:*v*) < methanol acetic acid 1 % (*v*:*v*) < butanol/DMF < chloroform/ethanol < MTBE/DMF < DMF acetic acid 1 % (*v*:*v*) < MTBE/DMSO < THF < ethanol < DMF < ethylene glycol < DMSO < pentane/ethanol < DMSO acetic acid 1 % (*v*:*v*). Table 2 below shows organic extracts whose antioxidant activity was greater to that of the aqueous extract.

With the TEAC and ORAC methods, only fifteen extracts had greater antioxidant activity than the aqueous extract. The most active extracts in TEAC were the same as in ORAC but with different radical scavenging activities depending on the method used. The highest antioxidant potential for radical scavenging was obtained with the THF extract in TEAC with 3183.0±16.0 µmol TE/g dry extract and the acidified DMSO extract in ORAC with 2683.5±11.7 µmol TE/g dry extract. The extracts obtained with solvent mixtures showed higher activity than those obtained using individual solvents except with ethanol, DMF, DMSO and THF. The antioxidant activity obtained in TEAC varied between 1474.1±4.4 and 3183.0±16.0 µmol TE/g dry extract; in ORAC between 1727.7±8.4 and 2683.5±11.7 µmol TE/g dry extract.

3.2. Cell viability results

Cell viability tests were performed only on organic extracts having a higher antioxidant activity compared to the aqueous extract. Toxicity tests were carried out on beta-pancreatic RIN5mF cells over 1 hour and 24 hours, which represents short and long term effects. The percentage of viability was calculated relative to the control cells incubated in RPMI-1640 culture medium alone. The results are shown in Figure 4.

After one hour of incubation, extracts did not show toxicity except that obtained from acidified methanol. The extracts obtained from the following solvent mixtures: butanol/acetonitrile; MTBE/DMF; methanol/acetic acid 0.1 % (*v*:*v*); pentane/ethanol and DMF/acetic acid 1 % (*v*:*v*) resulted in a lower viability at 70 % after 24 hours of incubation. The results obtained with extracts using methanol acetic acid 1% (*v*:*v*) and pentane/ethanol were statistically different from the control (aqueous extract), which showed no effect on cell viability.

4. Discussion

Selecting the appropriate solvent for the extraction of bioactive compounds from medicinal plants is a critical step in a bio-guided purification process. Indeed, there is generally a cocktail of compounds in the plants and, consequently, the selection of the solvent also means the selection of the products to be extracted. This has a direct impact on the observed biological activities. In the present study, several solvents and their mixtures were therefore used to extract as many compounds as possible. All tests were performed on 96-well microplates (Corning® Costar® 96-Well microplates, USA). The efficiency of the extraction solvent was evaluated by its activity potential obtained by physicochemical (TEAC, ORAC) and biological tests (cell viability).

Thus, of the 96 extracts obtained, only fifteen had greater antioxidant activity than that of the aqueous extract. In this study, they were generally extracts obtained from solvent mixtures belonging to different classes. Indeed, the use of solvent mixtures makes it possible to obtain solvents of intermediate polarities and thus to promote the extraction of several active compounds.

This can be explained by solvents selectivity, widening of the polarity range which allow a better solvation of the bioactive natural compounds. The same observation was made in the study of the polyphenols of *Lathyrus maritimus L* [31]. However, the extracts obtained from ethanol, DMF, DMSO and THF proved to be more active than certain extracts of solvent mixtures such as THF with an antioxidant potential up to 3183.0±16.0 µmol TE/g dry extract in TEAC and 2683.5±11.7 µmol TE/g dry extract in ORAC for DMSO acetic acid 0.1% (*v*:*v*).

Among the extracts having more antioxidant activity, were those obtained with polar aprotic solvents, hydrogen bonding solvents, electron pair donor solvents and mixtures of polar aprotic solvents and hydrogen bond donors. Aprotic solvents, due to their heteroatoms, have dipole moments that give rise to opposite mesomeric charges and favor intermolecular interactions. This is the case of DMF, DMSO and THF which gave higher antioxidant activities in this study. The use of this solvents type had also been proven in a study on black tea where DMF or her mixtures with another solvents also extracted more antioxidant compounds [32]. In this study, THF extract was more active in TEAC and that obtained with DMSO acetic acid 1% (*v*:*v*) was more active in ORAC (Table 2).

Table 2. TEAC and ORAC organic extracts compared to aqueous extract.

Extracts	TEAC (µmol TE/g dry extract)±SEM	ORAC (µmol TE/g dry extract)±SEM
THF	3183.0±16.0	2344.5± 29.8
DMSO / Acetic acid 1% (*v*:*v*)	2747.8±1.5	2683.5±11.7
DMSO	2570.7±7.0	2595.1±12.8
DMF / Acetic acid 1% (*v*:*v*)	2525.0±4.0	2277.0±15.5
DMF	2488.2±9.9	2441.9±15.2
Ethanol	2416.2±3.1	2373.6±5.7
Pentane / Ethanol	2404.1±9.6	2619.0±9.4
Ethylene glycol	2158.7±5.6	2541.4±13.0
MTBE / DMSO	2143.8±1.1	2292.5±15.6
Methanol / Acetic acid 1% (*v*:*v*)	2016.2±6.1	1962.9±23.1
MTBE / DMF	1816.2±2.4	2266.2±16.7
Butanol / Acetonitrile	1622.8±3.3	1784.5±28.3
Ethylene glycol / Acetic acid 1% (*v*:*v*)	1537.0±2.7	1857.4±11.3
Butanol / DMF	1504.6±2.6	1967.4±6.5
Chloroform / Ethanol	1487.0±5.2	2080.9±6.9
Water	1474.1±4.4	1727.7±8.4

In the case of ethanol, these results were in agreement with those of the study of the antioxidant potential of *Limnophila aromatica* roots [33]. Some discrepancies were noted between the TEAC and ORAC results. This can be explained by the different mechanisms of action of the two methods. Indeed, the TEAC method is direct and involves an electron transfer whereas the ORAC method is indirect and involves a proton transfer. Similar variations were observed in the berry study [34].

In several studies, ethanol had also proven to be a very good solvent for the extraction of polyphenols [35,36]. Cell viability tests showed that the extracts whose obtained with methanol acetic acid 1% (*v*:*v*) and pentane/ethanol were statistically different from the control (aqueous extract). This could be explained by the possible presence of toxic compounds, extractable specifically by these solvents. Fortunately, the aqueous extract showed no decline in viability, which confirms the wide traditional use of this plant in Senegal.

The antioxidant activity obtained and the toxicity tests made it possible to select the solvents to be used for the extractions of the active compounds in order to be able to test them on biological models. However, further studies are needed for the isolation and identification of antioxidant compounds and in vivo studies are also needed for a better understanding of their mechanism of action.

5. Conclusion

This study allowed the implementation of a new simple, fast and efficient method of selecting the appropriate solvent for the extraction of bioactive compounds from medicinal plants. It was shown that solvent mixtures are more efficient at extracting bioactive compounds than their corresponding individual solvents with the exception of ethanol, DMF, DMSO and THF. The proposed method should be extended to the study of other parts of plant and could be a method of choice to select the most effective solvent for extracting the active compounds.

Acknowledgments

The authors wish to thank the Ministry of Higher Education and Research of Senegal, Cheikh Anta Diop University of Dakar, Strasbourg University, the European Center for the Study of Diabetes in Strasbourg and the French Government for their material and financial support.

Disclosure statement

Conflict of interests: The authors declare that they have no conflict of interest.

Author contributions: All authors contributed equally to this work.
Ethical approval: All ethical guidelines have been adhered.
Sample availability: Samples of the compounds are available from the author.

ORCID

Mamadou Baldé
http://orcid.org/0000-0002-9917-9957
Saïd Ennahar
http://orcid.org/0000-0001-8987-7703
Stéphanie Dal
http://orcid.org/0000-0003-0870-6430
Séverine Sigrist
http://orcid.org/0000-0002-5766-2596
Alassane Wélé
http://orcid.org/0000-0002-7511-0832
Eric Marchioni
http://orcid.org/0000-0002-4002-642X
Diane Julien-David
http://orcid.org/0000-0002-0780-4947

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