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Evaluation of antimicrobial activity of selected plant extracts by rapid XTT colorimetry and bacterial enumeration

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Abstract

The aim of this study was to screen and evaluate the antimicrobial activity of indigenous Jordanian plant extracts, dissolved in dimethylsulfoxide, using the rapid XTT assay and viable count methods. XTT rapid assay was used for the initial screening of antimicrobial activity for the plant extracts. Antimicrobial activity of potentially active plant extracts was further assessed using the "viable plate count" method. Four degrees of antimicrobial activity (high, moderate, weak and inactive) against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, respectively, were recorded. The plant extracts of *Hypericum triquetrifolium*, *Ballota undulata*, *Ruta chalepensis*, *Ononis natrix*, *Paronychia argentea* and *Marrubium vulgare* had shown promising antimicrobial activity. This study showed that while both XTT and viable count methods are comparable when estimating the overall antimicrobial activity of experimental substances, there is no strong linear correlation between the two methods.

Keywords: Antimicrobial activity; DMSO plant extracts; Jordan; Viable plate count; XTT rapid assay

1. Introduction

The vast majority of modern medications were derived originally from ancient herbal traditions. Medicinal plants have been used for centuries as remedies for human diseases as they contain components of therapeutic value. There are numerous natural plant products which have antifungal, antibacterial and antiprotozoal activities that could be used either systemically or locally (Heinrich et al., 2004). Several plants containing volatile oils, polyphenols and alkaloids as active constituents are utilized as popular folk medicines, while others gained popularity in the form of finished products collectively named phytomedicines. During the second half of the 20th century, the acceptance of traditional medicine as an alternative form of health care and the development of microbial resistance to the classical antibiotics led researchers to investigate the antimicrobial activity of several medicinal plants. Antimicrobials of plant origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Iwu et al., 1999). The present study involves the systematic screening of Jordanian plants for antimicrobial activity as screened using rapid XTT colorimetry and bacterial enumeration. Some of the randomly selected plants, namely *Ballota undulata* and *Osyris alba*, have no recorded ethnobotanical usage in Jordan. For some of the studied plants, such as *Coridothymus vulgaris*, *Ononis natrix* or *Varthemia iphionoides*, antimicrobial activity has been reported using the agar diffusion method (Maruhenda and Gimenez, 1986; Afifi et al., 1991; Goren et al., 2003; Penalver et al., 2005). In the present study dimethylsulfoxide (DMSO) extracts of different parts of these selected plants were assessed for their antimicrobial activity.

2. Materials and methods

2.1. Microbial strains and growth conditions

Four different bacterial strains, *Pseudomonas aeruginosa* (ATCC 9027), *Escherichia coli* (ATCC 8739), *Staphylococcus*

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aureus (ATCC 6538P), and *Bacillus subtilis* (ATCC 6633) were used. Nutrient broth and agar were prepared according to the manufacturer's instructions. Batches of medium (25 ml in 100 ml Erlenmeyer flasks) were inoculated from fresh culture plates and incubated at 37 °C. All media were obtained from Oxoid (Basingstoke, UK).

2.2. Plant extracts

The specimens used for the present study were collected from different parts of Jordan. The taxonomical identification of the plants was done according to the published references and by Prof. Dr. B.E. Abu-Irmaileh, Faculty of Agriculture, University of Jordan. A voucher specimen from each plant specimen was kept at the Department of Pharmaceutical Sciences, Faculty of Pharmacy, University of Jordan. The names, parts used and common traditional applications of the examined plant species are given in Table 1.

2.3. Plant extracts' preparation

Plant samples collected during spring/summer 2005 or purchased in the same period were dried at room temperature and finely ground with a hammer mill. Each 2.5 g powdered plant material was extracted by refluxing with 25 ml ethanol for 30 min and kept overnight at room temperature before filtration. After filtration, ethanol was evaporated until dryness and the crude extracts were weighed. 0.1 g of the crude extract was dissolved in DMSO to a final stock concentration of 20 mg/ml. All extracts were kept at −20 °C until antimicrobial tests were carried out. Microbiological quality of the processed plants was monitored using the "streak plate" method, where incubated plates (37 °C) were monitored for any microbial growth for 7 days.

2.4. Phytochemical screening

Phytochemical screening by thin layer chromatography (TLC) was carried out only for plant species indicating promising antimicrobial activity by rapid XTT colorimetry. The DMSO extracts were subjected to TLC examination for group determination of the secondary metabolites. Modified Dragendroff's reagent for alkaloids, ferric chloride reagent for phenolics, Naturstoff reagent for flavonoids and vanilline/sulfuric acid reagent for steroids and terpenoids were used. Solvent systems for the development of ready coated analytical TLC plates (Merck) were selected according to Wagner and Bladt (1996).

2.5. Antimicrobial agents

Standard antimicrobial agents were used to measure the overall susceptibility of the bacterial test strains (amikacin

Table 1
List of the plant species screened for their antimicrobial activity using rapid XTT colorimetry

Plant name	Plant family	Parts used	Uses
Ajuga chia Schreber	Labiatae	H	Blood clotting, wound healing[a]
Apium graveolens L.*	Umbelliferae	L	Diuretic, carminative, tonic, aphrodisiac[a]
Arbutus andrachne L.	Ericaceae	L, St	Pectoral, diuretic, astringent, antiseptic, sedative[b]
Ballota undulata (Sieber ex Fresen.) Bentham	Lamiaceae	L	Not reported
Capparis spinosa L.	Capparaceae	L, St	Diuretic, rheumatic pain, astringent, hypoglycemic[a]
Coridothymus capitatus L.	Labiatae	L, F	Respiratory, digestive system and urinary tract diseases, inflammations[c]
Eleagnus angustifolia L.	Elaeagnaceae	L	Antipyretic, dysenthery, antidiarrheal[d]
Euphorbia hierosolyminata Boiss.	Euphorbiaceae	H	Laxative, digestive, externally in treatment of warts[b]
Euphorbia peplus L.	Euphorbiaceae	H	Diuretic, expectorant, laxative[a]
Hypericum triquetrifolium Turra	Guttiferae	L, St	Sedative, astringent, antidepressant, relieves spasm[a]
Lepidium sativum L.*	Crucuferae	L	Tonic, aphrodisiac, tuberculosis, cough, sterility[a]
Marrubium vulgare L.	Lamiaceae	L, F	Cough, tuberculosis, asthma, analgetic, liver cirrhosis[a]
Mercurialis annua L.*	Euphorbiaceae	L	Inflammation, rheumatism, antidiarrheal, externally in treatment of wounds[a]
Momordica balsamina L.	Cucurbitaceae	L, St	Antiviral, hypoglycemic[e]
Nepeta curviflora Boiss	Lamiaceae	F, L	Anemia, chills, antiflatulence, abdominal colics[a]
Ononis natrix L.	Leguminosae	H	Diuretic, antihypertensive, antibacterial[a]
Osyris alba L.	Santalaceae	L	Not reported
Paronychia argentea Lam.	Caryophyllaceae	F	Kidney stones, hypoglycemic[a]
Peganum harmala L.	Zygophyllaceae	L	Sedative, anticonvulsive, relaxant[b]
Reseda luteola L.	Resedaceae	Fr, L, F	Antidiarrheal, stomac ailments[a]
Rubia tinctorium L.	Rubiaceae	L	Antidiarrheal, wound healing, relieves spasm, diuretic, aphrodisiac, antiulacerogenic[a], antihemorrhoidal[b]
Ruta chalepensis L.	Rutaceae	L	Rheumatic pain, amenorrhoea, hysteria, vermifuge[a]
Teucrium leucocladum	Lamiaceae	L	Spasm, hypoglycemic[f]
Tilia cordata Miller	Tiliaceae	L	Antispasmodic, sedative[e]
Urtica urens L.	Urticaceae	L	Diuretic, antirheumatic, tonic, antispasmodic[a]
Varthemia iphinoides Boiss. & Blanche	Compositae	H	Common cold, stomach ailments[a]
Zizyphus jujube L.	Rhamnaceae	L	Astringent, vermifuge, antidiarrheal[a]

L: leaves, S: seeds, St: stems, F: flowers, Fr: fruits, R: roots, H: herb, *: fresh plant used. [a]Oran and Al-Eisawi, 1998; [b]Al-Khalil, 1995; [c]Ali-Shtayeh et al., 2000; [d]Lev and Amar, 2002; [e]El-Gengaihi et al., 1996; [f]Abu-Rmaileh and Afifi, 2000.

Table 2
Plant extracts screened for their antimicrobial activity by the XTT assay

Bacterial strains	Screened plant extracts showing antimicrobial activity [a,b,c]
Bacillus subtilis	H. triquetrifolium [a], *B. undulata* [a], *R. chalepensis* [a], *O. natrix* [a], *P. argentea* [a], *M. vulgare* [a]
Staphylococcus aureus	R. chalepensis [b], *O. natrix* [b]
Escherichia coli	O. natrix [c], *V. iphionoides* [c], *P. argentea* [c]

[a,b,c], high, intermediate and weak antimicrobial activity, respectively.

(Fluka), ampicillin (Fluka), cefuroxime (gift from Advanced Pharmaceutical industries Co/Jordan), chloramphenicol (Fluka), clindamycin (Sigma) and streptomycin (Sigma)). The abovementioned antimicrobials were dissolved in DMSO (Sigma) to obtain a stock concentration of 10 mg/ml.

2.6. Susceptibility assay using XTT colorimetry

The XTT colorimetry assay was performed according to Pettit et al. (2005) with some modifications. The sodium salt of XTT (Sigma) was dissolved in saline to give a concentration of 1 mg/ml. Menadione (Sigma) was dissolved in acetone to give a concentration of 1 mmol/l. The XTT/Menadione reagent was prepared freshly prior to each assay and contained 12.5 parts XTT/1 part Menadione. For bacterial culture preparation, 200 µl of overnight broth cultures adjusted to ca. 5×10^5 cells/ml in nutrient broth was added to 96-well flat bottom plate. Following a 17 h treatment of the 96-well plate cultures with 17 µl of plant extracts, antibiotic or DMSO (negative control), the plates were gently shaken in a shaker incubator (Boekel Scientific) and 100 µl from each well was transferred onto a new plate. Then to each well 25 µl of XTT/Menadione was added and the plates were gently shaken. After an incubation period of 1 h at 37 °C, the plates were again gently shaken and the absorbance at 490 nm was measured using a microplate reader (Universal Microplate Reader, ELx 800 UV). Blanks included media plus XTT/Menadione plus a plant extract or antibiotic concentration equal to that in the experimental wells. Percent reduction in formazan produced was calculated using the following formula (Pettit et al., 2005):

$$100\% - \left(\frac{\text{Experimental well absorbance at 490 nm} - \text{Blank absorbance at 490 nm}}{\text{Negative control absorbance at 490 nm}} \times 100\%\right)$$

Experimental substances were classified as having antimicrobial effect when they resulted in a mean ($n=2$) reduction in the XTT colorimetry assay of $\geq 50\%$ (threshold level for antimicrobial activity). A $<50\%$ reduction in XTT colorimetry assay was classified as either having little or no antimicrobial activity.

2.7. Susceptibility assay using bacterial enumeration

96-well plate cultures were prepared as described in Section 2.6. To this, 17 µl (340 µg/ml) of plant extract was added and incubated at 37 °C for 17 h. Pure DMSO (17 µl, 8.5% v/v) was used as negative control. Plates were gently mixed and 100 µl of the planktonic culture was taken to determine the number of the viable cells. The viable cells were determined by performing 10-fold serial dilutions of this suspension in 0.9% saline. All dilutions were plated on nutrient agar plates that were then incubated at 37 °C for 17 h. Incubated plates were further observed for a week. The antimicrobial activity of the tested plant extracts was compared to those of different standard antimicrobial agents (17 µl, 170 µg/ml) under similar culture conditions.

The activity of the experimental substances was neutralized by dilution effect and by mixing 100 µl of the resultant supernatant with 100 µl of 6% w/v tween 80 (Sigma), which was verified of having no antimicrobial effect.

Percent viable counting reduction was calculated using the following formula

$$100\% - \left(\frac{\text{Experimental well viable count}}{\text{DMSO well viable count}} \times 100\%\right)$$

In this study experimental substances exhibiting a mean ($n=3$) reduction in viable count of $\geq 90\%$ were classified as

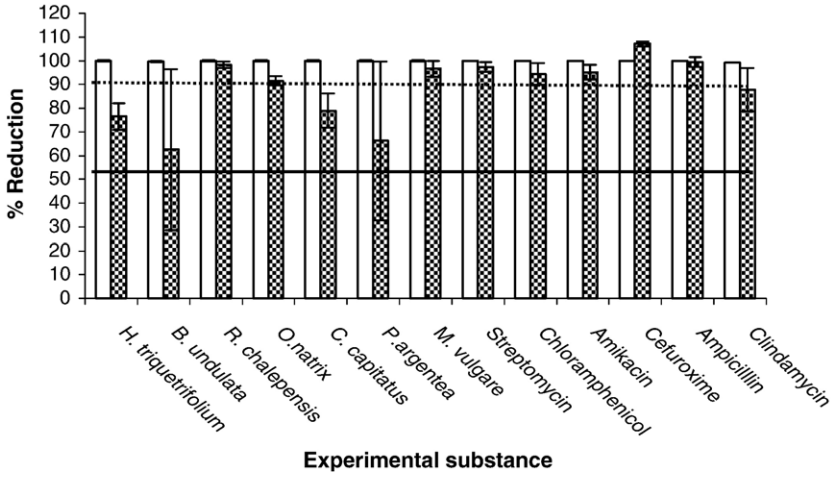

Fig. 1. The antimicrobial activity of experimental substances plant against the Gram positive bacteria *Bacillus subtilis* as assessed by percentage reduction in XTT colorimetry (solid bars) and percentage reduction in viable counting (open bars). Thresholds for minimum antimicrobial activity as assessed by reduction in percentage of XTT assay (−) and viable count (...) are shown. XTT data are mean ± S.D. values from two separate experiments. Viable count data are mean ± S.D. from three separate experiments.

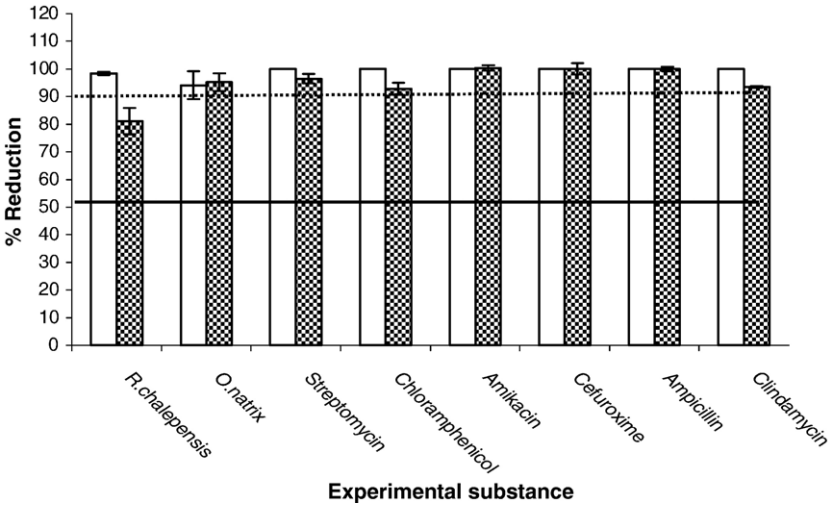

Fig. 2. The antimicrobial activity of experimental substances against the Gram positive bacteria *Staphylococcus aureus* as assessed by percentage reduction in XTT colorimetry (solid bars) and percentage reduction in viable counting (open bars). Thresholds for minimum antibacterial activity as assessed by reduction in percentage of XTT assay (−) and viable count (...) are shown. XTT data are mean±S.D. values from two separate experiments. Viable count data are mean±S.D. from three separate experiments.

having antimicrobial effect (threshold level for antimicrobial activity).

3. Results

Plant extract sterilization by autoclave is not possible due to the risk of possible heat degradation of the active compounds. DMSO extracts could not be filter-sterilized as DMSO dissolves the filter material. The DMSO dissolved plant extracts showed no microbial contamination by "streak plate" method. Studying the effect of DMSO on cell viability revealed that 7.8% (v/v) DMSO concentration was the optimum concentration at which no significant reduction in viable cell count of the tested microbial strains was detected compared to negative controls (no DMSO). Consequently, in order to detect a potential antimicrobial activity of the tested plant extracts, the concentration of DMSO used was 7.8% (v/v).

Selected plant species were initially screened for potential antimicrobial activity using the XTT colorimetry assay (Table 1). The antimicrobial activity of plant extracts was classified in accordance to a predefined threshold of a minimum of 50% reduction in the produced formazan (Table 2). From the tested 27 plants, only six plant species exhibited antimicrobial activity against two of the tested organisms, *B. subtilis* and *S. aureus* (Figs. 1 and 2). The antimicrobial activity of most of the tested plant extracts against *E. coli* was weak (Fig. 3). Weak antimicrobial activity was also exhibited against *B. subtilis* and *S. aureus* by different tested plant extracts (results not

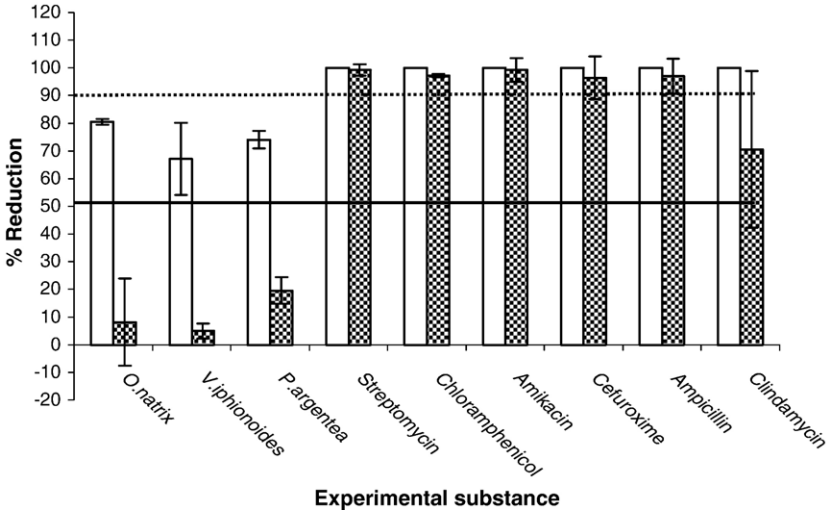

Fig. 3. The antimicrobial activity of experimental substances against the Gram negative bacteria *Escherichia coli* as assessed by percentage reduction in XTT colorimetry (solid bars) and percentage reduction in viable counting (open bars). Thresholds for minimum antibacterial activity as assessed by reduction in percentage of XTT assay (−) and viable count (...) are shown. XTT data are mean±S.D. values from two separate experiments. Viable count data are mean±S.D. from three separate experiments.

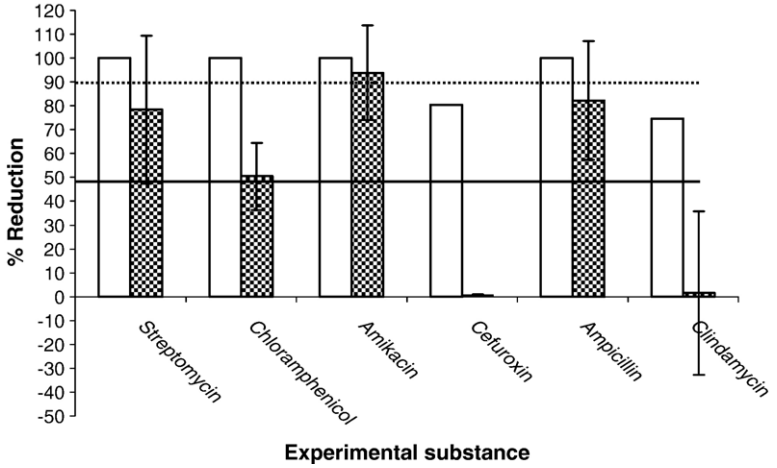

Fig. 4. The antimicrobial activity of experimental substances against the Gram negative bacteria *Pseudomonas aeruginosa* as assessed by percentage reduction in XTT colorimetry (solid bars) and percentage reduction in viable counting (open bars). Thresholds for minimum antibacterial activity as assessed by reduction in percentage of XTT assay (−) and viable count (...) are shown. XTT data are mean±S.D. values from two separate experiments. Viable count data are mean±S.D. from three separate experiments.

shown). No antimicrobial activity was recorded for any of the tested plant extracts against *P. aeruginosa* (Fig. 4).

Following the initial screening by XTT, the subsequent assessment of the antimicrobial activity by viable counting showed four different degrees of antimicrobial activity. These were graded as: high, moderate, low and no activity against *B. subtilis*, *S. aureus*, *E. coli* and *P. aeruginosa*, respectively. Plant extracts that were active against *B. subtilis* showed more than 6 log cycles reduction in viable count (99.9999%), while moderate reduction in viable count (ca. 95%) was seen against *S. aureus*. Less than one log reduction (ca. 70%) was seen against *E. coli* and no reduction in viable count was observed against *P. aeruginosa*.

The calculated Excel correlation function for the whole set of the experimental substances (plant extracts and antimicrobials) showed weak or no direct linear correlation between the two methods (correlation coefficient <0.5) when assessing the susceptibility of *B. subtilis* and *S. aureus*. Nevertheless, a strong positive correlation between the two methods, when assessing the antimicrobial activity against *E. coli*, was found (Table 3). The calculated correlation coefficients between the two methods for the standard antimicrobials against *B. subtilis*, *S. aureus* and *E. coli* were 0.7, 0.5 and 0.4, respectively.

The susceptibility of the tested bacterial strains to the different standard antimicrobials exhibited a high susceptibility of the Gram positive relative to the Gram negative bacteria. *P. aeruginosa* was the least susceptible among the tested bacterial strains as assessed by both methods (Figs. 1–4). The phytochemical screening of these plants showed the presence of flavonoids and phenolics in all the extracts. Terpenoids and alkaloids, on the other hand, were detected in five and two plant extracts, respectively (Table 4).

Table 3
Calculated Excel correlation coefficient between the percentage reduction in viable count and percentage reduction in XTT assay for the tested experimental substances

Tested bacterial strains	Excel correlation coefficient of % reduction in viable count vs % reduction in XTT absorbance
Bacillus subtilis	0.23
Staphylococcus aureus	0.23
Escherichia coli	0.94

4. Discussion

The conventional method used to assess the antimicrobial activity of different antimicrobial agents is by the determination of the minimal inhibitory concentration (MIC) either by broth dilution or by the agar diffusion method. These methods are well established procedures for which there are accepted guidelines including those endorsed by the National Committee for Clinical Laboratory Standards (NCCLS, 2003). Nevertheless, when examining the antimicrobial activity of poorly soluble experimental substances, solubilized in DMSO, the abovementioned methods are not suitable. Determination of MIC by agar diffusion necessitates a certain degree of hydrophilicity of the experimental substances. Furthermore, determination of MIC by the broth dilution method is not feasible since DMSO has a concentration dependent toxic activity against microbial strains, thus, interfering with the interpretation of results (Salvat et al., 2001). Accordingly, determination of the antimicrobial activity by MIC method

Table 4
Results of the TLC screening of the antimicrobial active plant species using rapid XTT colorimetry

Plant name	Alkaloids	Flavonoids	Terpenoids	Phenolics
Ballota undulata	−	++	−	+
Hypericum triquetrifolium	−/+	+	+	+
Marrubium vulgare	−	++	+++	++
Ononis natrix	−	++	−	+
Paronychia argentea	−	++	+	+
Ruta chalepensis	+	+++	++	++
Varthemia iphionoides	−	+++	+	++

might be misinterpreted, especially for experimental substances of low antimicrobial activity.

Hence, to overcome these drawbacks, the antimicrobial activity can be detected by performing viable counting following the exposure of cells to experimental substances. While this reveals the exact number of viable microorganisms, limitations arise with microbial strains tending to clump and having a slow growth rate such as mycobacteria (Millstein and Gibson, 1990). The use of potentially toxic solvents such as DMSO, ethanol, methanol, etc. requires that their maximum in use concentration should be predefined. Moreover, the time consuming and labor intensive nature of this method discourages its use as an initial screening method for large samples as in the case of plant extracts. Accordingly, a rapid, easy-to-use and inexpensive initial screening method with a high throughput potential would be of great value. The rapid and reliable XTT assay, a colorimetric method, fulfills these criteria. The XTT assay, nowadays, is extensively used for several purposes. These include: the quick determination of microbial strain susceptibility, evaluation of lot-to-lot consistency between vaccine batches and confirmation of the absence of live mycobacteria in tuberculin purified protein (Kairo et al., 1999; De Logu et al., 2003; Tunney et al., 2004; Cerca et al., 2005; Moriarty et al., 2005; Van de Sande et al., 2005).

Examining independent duplicate samples of the tested experimental substances by this rapid method was successful in the initial screening of plant extracts for their antimicrobial activity. Using this method, the antimicrobial activity is assessed by measuring the relative reduction in formazan production. The XTT assay indirectly measures the microbial activity by assessing the electron transport system (ETS) activity using artificial electron acceptors, redox dyes that can successfully compete with oxygen for electrons (Bensaid et al., 2000). The XTT [3′-[1-(phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzenesulfonic acid hydrate] is reduced by the dehydrogenase enzymes present in the ETS system to a water soluble formazan dye, the absorbance of which can be measured spectrophotometrically (McClusskey et al., 2005). This assay is valuable in providing a reasonable overall estimate of antimicrobial activity. The classical method of plate count was found to be suitable in defining the degree of antimicrobial activity (high, intermediate, low and inactive) of the different tested experimental substances.

While the XTT assay and viable count methods were comparable when estimating the overall antimicrobial activity of the different plant extracts, there was no linear relationship between both methods as recorded by the calculated correlation coefficient for *B. subtilis* and *S. aureus*. This poor correlation was reported in many studies and is based on the fact that microbial metabolic activity is not solely affected by the number of viable cells (Hatzinger et al., 2003; Honraet et al., 2005). Other factors such as the presence of certain chemicals that might cause an abiotic XTT reduction or formazan production inhibition will inevitably interfere with this assay (Bedwell et al., 2001; McClusskey et al., 2005). Moreover inter-strain differences and different modes of growth might also affect formazan production due to differences in metabolic activities (Kuhn et al., 2003; Honraet et al., 2005). On the other hand, a strong correlation was observed for *E. coli*. In general, the correlation coefficient depends on the variability in readings, where readings that differ greatly will produce larger correlation coefficients relative to more homogenous readings (Bland and Altman, 1996). This explains, in part, the high correlation coefficient in the case of *E. coli*. Nevertheless, the calculated correlation coefficient of standard antimicrobials (least variation and common types of tested material) did not show an excellent correlation (correlation coefficient >0.9) between the two methods. Accordingly, both methods are excellent for discriminating between two extreme antimicrobial effects (weak and high) but their capability to detect small differences in activity is small and probably less relevant.

The antimicrobial activity recorded for the crude plant extracts of *H. triquetrifolium*, *B. undulata*, *R. chalepensis*, *O. natrix*, *P. argentea* and *M. vulgare* against the tested Gram positive bacteria is promising. This antimicrobial activity is related to the different secondary metabolites; alkaloids, phenolics and terpenoids reported or identified in these plant species need further investigations. The antimicrobial activity of the tested plant extracts against the Gram negative bacteria was either low or inactive. In general, Gram negative bacteria show higher resistance towards antimicrobial agents and this is evident from the susceptibility results of *P. aeruginosa*.

In conclusion, we found that the rapid XTT method is a valuable method for screening of plant extracts for their antimicrobial activities. The antimicrobial activity of some plants might be considered sufficient for further studies aimed at isolating and identifying active principle(s) and evaluating possible synergism of antimicrobial activity among these extracts.

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