

REVIEW ARTICLE

Methods to Study the Phytochemistry and Bioactivity of Essential Oils

Mouhssen Lahlou*

Laboratory of Biochemistry, Cellular and Molecular Biology, Department of Biology, FSU: Biology and Health, Faculty of Sciences Ain Chock, Casablanca, Morocco

Many essential oils are extracted, analysed and their main components are identified, characterised and then published without any biological testing whatsoever. Their useful biological activities can remain unknown for years. Yet, the search for these activities often increases our knowledge of the potential use of oils in therapeutics. Therefore, there is a real need for a simple, reliable and reproducible methods to study the bioactivity of essential oils and their constituents which can detect a broad spectrum of action or specific pharmacological activities in aromatic plants. These methods can then be employed by natural product chemists, pharmacologists and biologists to conduct their scientific research and to valorise natural products. Standardisation of some of these methods is therefore desirable to permit more comprehensive evaluation of plant oils, and greater comparability of the results obtained by different investigators. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords: essential oils; phytochemistry; bioactivity; methodology.

INTRODUCTION

Essential oils are valuable natural products used as raw materials in many fields, including perfumes, cosmetics, aromatherapy, phytotherapy, spices and nutrition (Buchbauer, 2000). Aromatherapy is the therapeutic use of fragrances or at least mere volatiles to cure, mitigate or prevent diseases, infections and indispositions by means of inhalation (Buchbauer *et al.*, 1993a). This has recently attracted the attention of many scientists and encouraged them to screen plants to study the biological activities of their oils from chemical and pharmacological investigations to therapeutic aspects. Hopefully, this will lead to new information on plant applications and new perspective on the potential use of these natural products.

Many papers on the biological activity of essential oils have been published. The data, however, show much discordance between the same essence. The reasons for this variability can be understood if we take into account all the factors influencing the chemical composition of the oils, namely, climatic, seasonal and geographic conditions, harvest period and distillation technique, among others (Panizzi *et al.*, 1993). The effect of plant maturity at the time of oil production and the existence of chemotypic differences can also drastically affect this composition (Lahlou and Berrada, 2003). These variations are of distinct importance in

the study of biological and pharmacological activities of these products, as the value of an essential oil in aromatherapy has to be related to its chemical composition (Lawrence, 2000).

Most papers do not often cite the geographic origin and the exact composition of the essential oil studied, which somewhat negates their findings. No consideration is given to the basic question as to what the principles responsible for the therapeutic action are (Janssen *et al.*, 1987).

Essential oils are complex mixtures comprising many single compounds. Each of these constituents contributes to the beneficial or adverse effects of these oils. Therefore, the intimate knowledge of essential oil composition allows for a better and specially directed application (Buchbauer, 2000). Considering all the aforementioned differences in essential oil composition, it is clear that only a detailed knowledge of the constituents of an essential oil will lead to a proper use in cosmetics by perfumers and cosmetic chemists. However, such a detailed knowledge can only be obtained by means of carefully performed capillary-GC experiments (Buchbauer, 2000).

This paper is aimed primarily at summarising the phytochemical study and the bioactivity methods to investigate essential oils from aromatic plants as potential biological and pharmacological resources. Some consideration of the special, practical problems of testing volatile oils (poor water solubility) are also given, including suggestions for overcoming these problems. The study also provides a set of guidelines for research on biological, pharmacological and toxicological activities of plant oils with the aim of assessing whether an essential oil possesses a specific biological and pharmacological effect or whether it has a wider spectrum of action.

* Correspondence to: M. Lahlou, Laboratory of Biochemistry, Cellular and Molecular Biology, Department of Biology, FSU: Biology and Health, Faculty of Sciences Ain Chock, Km 8, POB 5366, Maârif 20101, Route of El Jadida, Casablanca, Morocco.
E-mail: lahloumoh@hotmail.com

PHYTOCHEMISTRY

Plant collection

There is often a lack of information on the distribution of the oil in different plant parts. Such information may have predictive value, but is not available due to the failure of most investigators to systematically study all parts of plants. For this reason, the investigated parts richer in essential oil (cones, fruit, leaves, bark) should be selected on a rational scientific basis and with solid justification (preliminary chemical screening). Furthermore, plants showing an aromatic character should be collected. Examples of such plant families are Anacardiaceae, Apiaceae, Asteraceae, Chenopodiaceae, Cupressaceae, Gentianaceae, Lamiaceae, Myrtaceae, Pinaceae, Piperaceae, Poaceae, Rutaceae, Verbenaceae and Zingiberaceae.

Essential oil extraction

'Essential oils are products, generally, of rather complex composition comprising the volatile principles contained in the plants, and more or less modified during the preparation process' (Bruneton, 1995). They are essentially obtained by hydrodistillation (the plant material is heated in two to three times its weight of water with indirect steam from outside the still) as opposed to steam distillation (the plant material is extracted by direct steam, produced in the still, or by indirect steam, produced outside and fed into the still), hydrodiffusion (low-pressure steam (< 0.1 bar) replaces the volatile from the intact plant material by osmotic action) or CO₂ extraction (Buchbauer, 2000); in addition to expression of the pericarp (or cold pressing) which is a special method for *Citrus* (Rutaceae) peel oils extraction (Baaliouamer *et al.*, 1992; Dellacassa *et al.*, 1992) from fresh or dried material.

The microwave irradiation [or microwave assisted process (-MAP-)] has also been developed and reported by many authors as a technique for extraction of essential oils in order to obtain a good yield of the essence and to reduce time of extraction (Paré *et al.*, 1989; Collin *et al.*, 1991; Bouzid *et al.*, 1997; Brosseau, 1997; Chiasson *et al.*, 2001; Ghouliami *et al.*, 2001). This technique has also been applied for the extraction of saponins from some medicinal plants (Safir *et al.*, 1998). The MAP process uses microwaves to excite water molecules in the plant tissues causing plant cells to rupture and release the essential oils trapped in the extracellular tissues of the plant (Bélanger *et al.*, 1991).

Another technique consists of extracting oils using a mechanical and thermochemical reaction (Bouzid *et al.*, 1997). Yield data of these oils is therefore determined on a fresh weight basis (w/v).

Chemical analysis

Chemical analysis of essential oils is generally performed using GC (quantitative analysis) and GC/MS (qualitative analysis) (Keravis, 1997). Identification of the main components is carried out by the comparison of both the GC retention times and MS data against those of

the reference standards (with known source); as previously reported (Lahlou *et al.*, 2000b; 2001b,d; Lahlou and Berrada, 2003).

Analytical conditions and procedures used should carefully be described. These included:

- apparatus of oil analysis (make and model number of the equipment);
- column type and dimensions;
- carrier gas flow rate;
- the temperature programming conditions including injector temperature, detector and column temperatures; in addition to mass spectra (electronic impact).

Sometimes identification by GC/MS must be confirmed by retention indices (Kovats Indices) on two columns of different polarity, as reported in our earlier work (Lahlou, 2003); or on the same column, but at a different temperature, as indicated by Denayer and Tilquin (1994); and claims for the identification of new constituents should be supported by co-injection with authentic compounds. Data should thus include essential oils optical rotation, density and refraction index (Lahlou *et al.*, 1999; 2000a).

On the other hand, compounds which are not easily separated by GC, and molecules structurally similar like stereo-isomeric compounds of essential oils are analysed by ¹³C NMR as reported by Tomi *et al.* (1997). This technique is also applied to the study of the chemical intraspecific variation and could also be used in the quality control of volatile oils.

BIOACTIVITY PROCEDURE

Animal models for pharmacological and toxicological studies

Many countries and organisations have legislation and guidelines for the care and use of animals for pharmacological and toxicological research purposes (Guide for the Care and Use of Laboratory Animals, 1985; International Guiding Principles for Biomedical Research Involving Animals, 1985; Interdisciplinary Principles and Guidelines for the Use of Animals in Research, 1988; Use of Laboratory Animals in Biomedical and Behavioural Research, 1988). Moreover, it is the responsibility of researchers to ensure that their practices conform with those relevant to them. Training and research involving animals should incorporate procedures which are designed and performed with due consideration of current scientific knowledge, the relevance to human or animal health, the advancement of the science of toxicology and the potential benefit to society. Nevertheless, these researches must contain precise details on animals, especially the choice of the adequate laboratory animal model for a specific pharmacological study. Thus, the species, (e.g. *Psammomys obesus* rats, *Meriones shawi*, Wistar rats, hamster, dogs, monkeys, rabbits, Swiss mice, Sprague-Dawley), should be carefully selected and the number of animals kept to the minimum required to achieve reproducible and scientifically valid results. Experiments that require the use of animals must be conducted in accordance with the Guiding Principles in the Use of Animals in

Toxicology, which were adopted by the Society of Toxicology in 1989.

Dispersion and solubility of essential oils

Generally, essential oils are poorly soluble in water, and this causes many problems for studying their biological and pharmacological properties. In order to overcome these problems, many authors have recommended the use of various solvents in the dilution of essential oils such as acetone, alcohol, ethylene glycol, ethanol, methanol, DMSO and DMF (Dayal and Purohit, 1971; Allegrini et Simeon de Buochberg, 1972; Martinez Nadal *et al.*, 1973; Beylier-Maurel, 1976; Morris *et al.*, 1978; Conner and Beuchat, 1984; Lahlou *et al.*, 2000b); or using an emulsifier detergent or 'Tensioactif' like Tween 20 or Tween 80 in different percentage (Allegrini et Simeon de Buochberg, 1972; Allegrini *et al.*, 1973; Pellecier *et al.*, 1976; Beylier, 1979; Benjilali *et al.*, 1984; 1986; Lahlou *et al.*, 1999; 2000a; 2001a,b; Lahlou, 2003). Furthermore, Chalchat *et al.* (1991) compared the results obtained for the determination of the MIC of essential oil constituents using two modern methods in liquid media, one involving Tween 80 as emulsifier and the other using noble Agar as stabiliser. The authors concluded that the two methods gave comparable results.

On the other hand, Remmal *et al.* (1993a,b; 2001) demonstrated that ethanol, Tween 80, Tween 20 and Triton X100 present a depress effect of antimicrobial activity of tested oils in a solid medium, in addition to their antagonist effect in a liquid medium. The authors suggested the use of 0.2% of agar suspension. In this case, minimal inhibitory concentration (MIC) and minimal lethal concentration (MLC) found by these authors for different bacterial species in the presence of agar were significantly lower than those observed in the presence of Tween 80 or ethanol. This demonstrates the fact that solvents and detergents often used in antimicrobial studies significantly decrease the antibacterial activity of tested oils. The use of agar suspension to disperse essential oils also resulted in obtaining lower MIC (Lens-Lisbonne *et al.*, 1987) than those previously reported by Morris *et al.* (1978) and Simeon de Buochberg (1976) for the same micro-organisms and the same oils dispersed with ethanol and Tween 80, respectively.

Balansard (1990) and Remmal *et al.* (2001) proposed another method to standardise emulsion of essential oils essentially for antimicrobial tests in order to maintain their viscosity stable in aqueous medium. This technique consists of adding 50 to 100 µl of oil to a sterile tube containing 10 ml of noble agar. After centrifuge agitation, the exact quantity of oil was determined after tube weighing; the volume of agar solution was calculated in order to avoid desired concentrations of the emulsion (2 to 4 mg/ml). These emulsions were easily used without particular technical problems for all evaluation of tested oils.

The study undertaken by Santos *et al.* (1997) on antibacterial activity of some essential oils did not utilise Tween 80 (detergent) or ethanol (alcohol) to disperse the essential oils with agar as these have been reported to interfere with the estimation of antimicrobial activity of essential oils (Remmal *et al.*, 1993b). The dispersion

with agar was considered homogeneous as a true solution in ethanol. Furthermore, MIC (minimal inhibitory concentrations) for different bacterial species in the presence of agar appears to be significantly lower than those observed in the presence of Tween 80 or ethanol (Remmal *et al.*, 1993b).

Laboratory conditions and experimental techniques

Results of biological tests of essential oils are often different and dependent upon experimental conditions per comparison to the literature data. These are both related to the laboratory conditions (temperature, photoperiod, etc.), materials; in addition to the models taken for biological experimentation (age, life cycle). Moreover, the method of extraction of essential oils, influences their chemical composition, and thus, can have repercussion regarding their biological properties. Techniques of solubility of these oils are among others problems. Normalisation of methods and laboratory conditions, in addition to the techniques used (extraction, dissolution and dispersion of oils, culture medium) for testing biological and pharmacological activities of essential oils at the definitive stage of laboratory screening are desirable in order to provide a common basis for the comparison of results obtained in various parts of the world on different organisms tested under similar laboratory conditions.

Whenever pharmacological bioassays are investigated in persons presenting with some heart-vascular diseases (hypertensive diabetes) or in healthy volunteers (for the study of the effects of fragrances on autonomic nervous system parameters and self-evaluation, for example), studies in these cases should take into account sex, age, weight and socio-cultural level of persons who took part in the experiments, in addition to the number of subjects, which allows homogeneous statistical analysis. Furthermore, experiments should always take place at the same time of day (the same conditions). Sometimes, 24 h prior to the beginning of each experiment, subjects had to abstain from food and beverages containing the substances tested. Moreover, all subjects should give written informed consent to all aspects of the study. A second group of subjects should be used as a control.

Correction of percentage mortality

Lethal effect of essential oils or their components on biological organisms should take into account mortality in the control. Thus, in cases where control deaths occurred, the data should be corrected using Abbot's (1925) formula, as reported in our works (Lahlou *et al.*, 2000b; 2001d; Lahlou, 2002; Lahlou and Berrada, 2003).

Statistical analysis

Lethal concentrations/doses LC50, LC90 (or lethal doses LD50, LD90) and 95% confidential limits could be determined according to Dupont (1970) using the method of Litchfield and Wilcoxon. They can also be calculated using the Reed and Muench method (1938)

or using probit analysis method as described by Finney (1971), as reported in our works (Lahlou, 2001; Lahlou *et al.*, 2001a,b,c,d; Lahlou, 2002; 2003; Lahlou and Berrada, 2003; Lahlou *et al.*, 2003).

Lethal doses LD₅₀ could also be calculated using the INRA-INSA 'Toxicologie' analysis software which uses the probit method developed by Bliss (1935). The results of biological activities could be presented as mean \pm SEM, with *n* indicating the number of observations. Values can be analysed using a Student's *t*-test, ANOVA or non-parametric tests, where appropriate, and are considered to differ significantly when *p* < 0.05.

In cases where data are insufficient for these techniques, the dose-response data should be transformed into a straight line by means of a logit transformation (Swaroop, 1966); lethal concentrations are derived from the best fit lines obtained by linear regression analysis.

Results of other pharmacological or physiological activities are usually expressed as means \pm the standard error of the mean used in order to construct a dose-response curve. The significance of the results should be assessed by means of unpaired or paired Student's *t*-tests, Mann-Whitney U-tests, Dunn's test and one-way (doses) or two-way (treatment \times doses) analysis of variance (ANOVA) (Lahlou *et al.*, 1999; 2000a).

Factors affecting the bioactivity of essential oils

Studies of biological and pharmacological activities of essential oils/constituents may take into account the following parameters and factors:

Effect of treated plant part. In order to indicate a difference in activity between two different parts of the same plant, many studies have been performed. In particular, for molluscicidal tests (Lahlou *et al.*, 2001b), oil obtained from leaves of *Citrus aurantium* var. *amara* Link (Rutaceae) was devoted on activity to *B. truncatus* snails at tested concentrations. Whereas, oil of the fruit of the same plant exhibited potent molluscicidal activity at lower concentration (LC₅₀ = 1.46 (1.36–1.56) ppm).

In fact, there is a lack of information on the distribution of the biological activity in different plant parts essentially related to the difference in distribution of active compounds (or active principles) which are more frequent in some plant parts than in others. Although many other compounds do occur independently, difference in chemical composition of these oils and gives a rise to these observations.

Effect of the solvent/detergent. The most useful solvents in laboratory are toxic to biological organisms and consequently interfered with the activity being studied (Lahlou, 2001). The choice of an adequate solvent (or detergent) is indispensable before envisaging a bioassay using an essential oil. Thus, in order to demonstrate the effect of the detergent (quantity), used for dispersion of an essential oil on the results of biological tests, a comparative study was carried out for the same 'tensio-active' Tween 80 when used differently in two different pieces of research on *Bulinus truncatus* snails (Lahlou *et al.*, 2001a,b). In this case two very different sets of results were obtained.

In order to facilitate the essential oils' dispersion in the aqueous medium, the Tween 80 was used at 1.0% (Lahlou *et al.*, 2001a). The results obtained indicated an interesting molluscicidal activity of these compounds at much high concentrations ($0.20 \cdot 10^3 \text{ ppm} \leq \text{LC}_{50} \leq 2.85 \cdot 10^3 \text{ ppm}$ for oils and $0.12 \cdot 10^3 \text{ ppm} \leq \text{LC}_{50} \leq 2.68 \cdot 10^3 \text{ ppm}$ for the main components). When the Tween 80 was used at 0.2% only, and the solutions obtained were then dispersed in water by application of 2KC/s ultra-sound frequency for 5 sec, milky clear solutions were obtained and various concentrations were prepared. The results obtained from this second technique (Lahlou *et al.*, 2001b) are high enough in comparison to that of the latter and tested oils and their constituents acted at low concentrations ($0.28 \text{ ppm} \leq \text{LC}_{50} \leq 2.84 \text{ ppm}$ for oils and $0.13 \text{ ppm} \leq \text{LC}_{50} \leq 2.93 \text{ ppm}$ for the main components).

Thus, the technique used for dissolution of tested oils and their main components using a minimum quantity of the 'tensio-active' Tween 80 (0.2% only) in addition to the physical method for their dispersion (ultra-sound apparatus), has led for the first time to a good solubility, diffusion and dispersion of the essential oil in water, thus ensuring a better contact of these oils and/or their constituents with tested organisms (snails in this case). Therefore, the quantity of Tween 80 used for dispersion of an essential oil had an important influence on its biological activity.

The correlation composition-activity. Biological activity of an essential oil is in strict direct relation to its chemical composition. Thus, in our screening plants used in local folk medicine for biological properties (Lahlou *et al.*, 2000b; Lahlou, 2001; Lahlou *et al.*, 2001a,b,d; Lahlou, 2002; 2003; Lahlou and Berrada, 2003), we noted a difference in activity of tested oils and their main components on different biological models studied. Essential oil, in its totality, acted less than the major constituents. In these studies, and in order to search for the active components responsible for the biological activity, we studied the main components of oils that have shown toxicity to these biological models. The relation between composition and activity leads us to suggest in these cases that biological activity of the essences from the aromatic plants studied may be attributable both to their major components (alcoholic, phenolic, terpenic or ketonic compounds) and to the minor ones present in these oils. It is possible that they may act together synergistically to contribute to the toxicity of the totality of the tested oil.

Technique used. During our *in vitro* study for licial and niticidal activities of some Moroccan essential oils and their main components or plant oil chemotypes on *Pediculus humanus capitis* (head lice), two methods were investigated: microatmosphere and direct application (Lahlou *et al.*, 2000b; Lahlou and Berrada, 2003). It was found that some oils tested were most active on lice using the microatmosphere technique [*Chenopodium ambrosioides* (Chenopodiaceae), *Mentha pulegium* and *Thymus broussonettii* (Lamiaceae) (LD₁₀₀ 1 h = 3.10 μ l)]. Whereas others were found to be much less active using this method [essentially *Lavandula stoechas* (Lamiaceae), *Chrysanthemum viscidhirtum* (Compositae), and *Cedrus atlantica* (Pinaceae)]. While,

during niticidal tests and using the microatmosphere way, essential oils from *C. ambrosioides*, *M. pulegium*, *T. broussonettii* and *O. compactum*, in addition to *Ruta chalepensis* were found to be the most potent among those tested ($LD_{100} = 3.10 \mu\text{l}$), other oils such as those from *Citrus limon* and *C. sinensis* (Rutaceae), *Pinus pinea* (Pinaceae), *Myrtus communis* (Myrtaceae), *Cinnamomum zeylanicum* (Lauraceae), *Pelargonium setaceum* and *Chrysanthemum viscidifolium* (Compositae) acted more potently using the microatmosphere technique (10–30% of nits hatched) than in direct application (0% of nits hatched). Essential oil from *Melaleuca viridiflora* (Myrtaceae) was more active on nits essentially in direct application (0% of nits hatched), whereas 30% of nits hatched in microatmosphere of $3.10 \mu\text{l}$ using this oil.

Biological material. During our study on licicidal and niticidal activities of some Moroccan essential oils (Lahlou *et al.*, 2000b), we observed that those derived from *Mentha pulegium* and *Thymus broussonettii* (Lamiaceae), *Chenopodium ambrosioides* (Chenopodiaceae), and *Ruta chalepensis* (Rutaceae) possess the most powerful licicidal and niticidal activities towards head lice, *Pediculus humanus capitis*. Other oils were found to be active only on nits like *Origanum compactum* (Lamiaceae) until volume of $3.10 \mu\text{l}$. On the other hand, using the direct application technique, lice were found to be more sensitive and died within 15 min of exposure to an application of $0.5 \mu\text{l}$ of all tested oils.

Choice of the doses/concentrations. Paracelsus' statement remains true down the ages: 'All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and remedy' (Paracelsus, pp. 1493–1541). Thus, it is important to note that this choice must not be hazardous. In addition, this character is most important for the study of bioactivity. Furthermore, these concentrations should be in accordance with the limits already documented in the literature (other works for identical activity using the same compounds or identical kind of plant). For this reason, in all our tests performed on biological organisms, when an essential oil or the main components tested was found to be toxic at a concentration, other appropriate lower concentrations (or dilutions of active ones) were prepared and performed in order to find the minimal toxic concentration which caused 50% or 90% toxicity or lethal doses/concentrations LD_{50} - LD_{90} / LC_{50} - LC_{90} . For example, in niticidal and licicidal tests (Lahlou *et al.*, 2000b; Lahlou and Berrada, 2003), different volumes of the oils (3.10 – $50 \mu\text{l}$) were tested.

Since essential oils are highly concentrated fluid substances, they are rarely used in an undiluted form. Before application, oils are first blended with a carrier oil. This blending dilutes the essential oils so that they are safe, and also helps to slow down the rate of evaporation, to spread them evenly, and to increase their eventual absorption into the skin. For this reason, active niticidal oils obtained from *Chenopodium ambrosioides* (Chenopodiaceae), *Mentha pulegium*, *Thymus broussonettii* and *Origanum compactum* (Lamiaceae), in addition to *Ruta chalepensis* (Rutaceae) were tested in diluting the low potent volume ($3.10 \mu\text{l}$) at dilution factors of 1:2, 1:4, and 1:10 in alcohol 95° (Lahlou *et al.*, 2000b).

The perfume concentration in most cosmetic products ranges between 0.5% and 2%, thus being too small for any risk of toxicity from essential oil-constituents, with the exception of some more or less toxic terpenic ketones, such as thujone or pulegone (Buchbauer, 2000).

Reference compounds

All biological and pharmacological tests using natural products must include proper controls, and in this respect, comparison with an agreed reference standard compound, under similar laboratory conditions, is essential and indispensable. It is recommended that this standard should be as follows (Table 1).

Comparison with literature data

This step is important in order to conduct a scientific laboratory research with a rigorous procedure in order to compare all findings with those reported in the literature for other compounds studied under identical laboratory conditions. This also indicates whether the results found are within the accepted ranges (or limits) as reported in the literature or are more or less interesting. Activity of oils from aromatic plants of the same family are rigorously comparable. However, there is some difficulty related to the conditions of experimentation which means that the results are sometimes relative. In general, using the same plant species oil should result in similar biological or pharmacological activities under identical laboratory conditions around the world. Moreover, it is necessary to check in the literature to ascertain if tested plant oils have been previously investigated for the same properties.

METHODOLOGY

Antiparasitic activities

Molluscicidal activity. Schistosomiasis is a parasitic disease in most developing countries. Fresh water snails act as the intermediate host; so control of these diseases is best achieved by breaking the transmission cycle, either by avoidance of infected water or destruction of the intermediate host, the snails (Lahlou *et al.*, 2001b). Bioassays were performed using *Bulinus truncatus* snails according to our works (Hmamouchi *et al.*, 1998, 2000; Lahlou, 2001; Lahlou *et al.*, 2001a,b; 2002; Lahlou, 2003). Thus, five snails of uniform size (4–6 mm shell height) were tested in distilled water containing each concentration of tested product. The exposure time was 24 h followed by a recovery period of 24 h. Death of snails was ascertained by examining immobilised snails under a dissecting microscope for the absence of heart beat. Tests were performed in triplicate.

Licicidal and niticidal activities. Tests on head lice, *Pediculus humanus capitis* and their nits were performed in identical Pyrex glass Petri dishes (5.5 cm diameter, 1.5 cm height) according to our works (Lahlou *et al.*, 2000b; Lahlou and Berrada, 2003), using two techniques:

Table 1. Standard compounds recommended for biological and pharmacological tests

Biological/Pharmacological tests	Standard compounds (controls)	References
Molluscicidal and cercaricidal	Niclosamide (bayluscide)	WHO, 1970; Gebremedhim <i>et al.</i> , 1994; Lahlou, 2001; Lahlou <i>et al.</i> , 2001a,b,c; Lahlou, 2003
Insecticidal and larvicidal	Temephos, abate, malathion, allethrin, deltamethrin, bromophos, fenitrothion, fenthion, chlorpyrifos	WHO, 1981; Laurent <i>et al.</i> , 1997; Jantan <i>et al.</i> , 1999; Lahlou, 2001; Lahlou <i>et al.</i> , 2001d; Lahlou <i>et al.</i> , 2003
Antibacterial	Streptomycin sulphate, cephalosporin C, penicillin G, gentamicin, tetracycline, actinomycin D, rifampicin, ampicillin, piperacillin, kanamycin, chloramphenicol, streptomycin, tobramycin, enrofloxacin, benzyl penicillin, cloxacillin, ciprofloxacin, cephalzoline	El Mahi <i>et al.</i> , 1997; Santos <i>et al.</i> , 1997; Larhsini <i>et al.</i> , 1999; Akinpelu and Olorunmola, 2000; Khan <i>et al.</i> , 2000; Takaisi-Kikuni <i>et al.</i> , 2000; Ebi, 2001; Erdemoglu and Sener, 2001; Marquina <i>et al.</i> , 2001; Ogunwande <i>et al.</i> , 2001; Rojas <i>et al.</i> , 2001; Schlemper <i>et al.</i> , 2001; Atindehou <i>et al.</i> , 2002; Chowdhury <i>et al.</i> , 2002; Asha <i>et al.</i> , 2003; Chowdhury <i>et al.</i> , 2003; Fleischer <i>et al.</i> , 2003a,b; Gupta <i>et al.</i> , 2003; Copland <i>et al.</i> , 2003; Kumarasamy <i>et al.</i> , 2003a,b; Omer and Elnima, 2003; Perez <i>et al.</i> , 2003; Somchit <i>et al.</i> , 2003
Antifungal/fungicidal/antimycotic	Griseofulvin, ampicillin, amphotericin B, chloramphenicol, econazole, nystatin, sulconazole, itraconazole, miconazole, 5-flucytosine, propiconazole, tioconazole, fluconazole, ketoconazole, benlate, nabam, clotrimazole, uconazole, bifonazole	Testa <i>et al.</i> , 1991; Rahalison <i>et al.</i> , 1994; El Mahi <i>et al.</i> , 1997; Akinpelu and Olorunmola, 2000; Khan <i>et al.</i> , 2000; Ebi, 2001; Erdemoglu and Sener, 2001; Kariba <i>et al.</i> , 2001; Marquina <i>et al.</i> , 2001; Rojas <i>et al.</i> , 2001; Rub Nawaz <i>et al.</i> , 2001; Ogunwande <i>et al.</i> , 2001; Chowdhury <i>et al.</i> , 2002; 2003; Lahlou <i>et al.</i> , 2002; Fleischer <i>et al.</i> , 2003a,b; Panagouleas <i>et al.</i> , 2003; Perez <i>et al.</i> , 2003; Somchit <i>et al.</i> , 2003; Omer and Elnima, 2003; Vollekov <i>et al.</i> , 2003
Antiparasitic	Praziquantel, santonin, pyrantel pamoate, mebendazole, piperazine citrate, biltricide, metronidazol (Flagyl®), albendazole, levamosol, gentian violet metronidazole, pentamidine, sodium stibogluconate (Pentostam [®]), chloroquine, emetine, sulfamonomethoxine, pyrimethamine	Keita <i>et al.</i> , 1990; Awad and Probert, 1991; Ndamba <i>et al.</i> , 1994; Martin <i>et al.</i> , 1997; Munoz <i>et al.</i> , 2000; Ishih <i>et al.</i> , 2001; Teixeira <i>et al.</i> , 2001; Shuhua <i>et al.</i> , 2000; Onyeyili <i>et al.</i> , 2001; Isah <i>et al.</i> , 2003; Tapia-Pérez <i>et al.</i> , 2003
Glycemic/antidiabetic	Dao nil (glibenclamide), streptozotocin, sulfamid, biguanid, metformin, tolbutamide	Chattopadhyay <i>et al.</i> , 1993; Skim <i>et al.</i> , 1999; Kameswara <i>et al.</i> , 2001; Chakrabarti <i>et al.</i> , 2003; Ohiri <i>et al.</i> , 2003
Gastrointestinal disorders/ Gastroprotective	Atropine, dicyclomine, neostigmine, nordihydroguareitic acid, cimetidine	Santos and Rao, 2001; Baggio <i>et al.</i> , 2003
Antitussive	Codeine phosphate	Boominathan <i>et al.</i> , 2003
Hypotensive	Propranolol, nifedipine	Lahlou <i>et al.</i> , 2000a
Central inhibitory	Nitrazepam, diazepam	Amos <i>et al.</i> , 2003
Relaxant effect	Theophylline	Boskabady and Ramazani-Assari, 2001; Boskabady and Khatami, 2003
Immunomodulatory	Cyclophosphamide, D-penicillamine	Gokhale <i>et al.</i> , 2003
Antipyretic	Paracetamol	Gupta <i>et al.</i> , 2003
Anti-inflammatory	Phenylbutazone, carrageenin, indomethacin, dichlorfenac, acetylsalicylic acid, piroxicam	Susplugas <i>et al.</i> , 1993; Choi <i>et al.</i> , 2000; Muko and Ohiri, 2000; Badilla <i>et al.</i> , 2003; Dongmo <i>et al.</i> , 2003; Mandal <i>et al.</i> , 2003; Medhi <i>et al.</i> , 2003; Sayyah <i>et al.</i> , 2003
Diuretic	Diamox, furosemid	Rhiouani <i>et al.</i> , 1999
Anti-stress	Nimodipine	Choi <i>et al.</i> , 2001
Sedative	Sodium thiopental, diazepam	Rakotonirina <i>et al.</i> , 2001
Antinociceptive	Indomethacin, acetylsalicylic acid	Badilla <i>et al.</i> , 2003; Rahman <i>et al.</i> , 2003
Antidiarrhoeal	Atropine sulfate, diphenoxylate, chlorpromazine, loperamide, dicyclomine	Chaves <i>et al.</i> , 1998; Biswas <i>et al.</i> , 2002; Sairam <i>et al.</i> , 2003; Hajhashemi <i>et al.</i> , 2000
Antitumour/anticancer	Cyclophosphamide	Lamchouri <i>et al.</i> , 1999
Analgesic	Morphine, paracetamol, acetylsalicylic acid, lorazepam, indomethacin, aspirin	Susplugas <i>et al.</i> , 1993; Alaoui <i>et al.</i> , 1998; Aziba <i>et al.</i> , 2001; Sayyah <i>et al.</i> , 2003

- (i) *Microatmosphere*: in this method, the whole oil or component evaporates into the atmosphere of a closed Petri dish, saturating it with vapour, whereby the volatile oil can exert an inhibitory effect on lice and hatching nits.
- (ii) *Direct application*: in this technique, 0.5 µl of the essential oil or one of their main components was applied directly onto the nits and lice and the results observed.

Antiprotozoal oils. Mikus *et al.* (2000) examined 12 essential oils and eight terpenes for antiprotozoal activity. They were screened for antitrypanosomal and antileishmanial activity by an *in-vitro* growth inhibition assay, whereby the protozoa were seeded into wells containing different concentrations of the test substances. After allowing 72 h for growth, efficacy was evaluated colorimetrically via an oxidation-reduction indicator and expressed as the ED50 (effective dose), the inhibitory concentration that reduced the growth rate by 50%.

Antimicrobial tests

In recent years due to an upsurge in antibiotic-resistant infections, the search for new prototype drugs to combat infections is an absolute necessity and in this regard plant essential oils may offer great potential and hope. These products have frequently been reported to be antimicrobial agents (Martinez Nadal *et al.*, 1973; Franchomme, 1981; Benjilali *et al.*, 1984, 1986; Tantaoui-Elaraki *et al.*, 1992; 1993; Panizzi *et al.*, 1993; Remmal *et al.*, 1993a; Remmal, 1994; Chanegriha *et al.*, 1994; Lattaoui *et al.*, 1994; 1997; Lacoste *et al.*, 1996; Tantaoui-Elaraki, 1997). According to the latter author, the study of antimicrobial activity of essential oils presents some difficulty summarised as follow:

- (i) essential oils present a complex chemical composition;
- (ii) they are poorly soluble in water like their constituents;
- (iii) some of their constituents are volatile;
- (iv) they must be used in low doses.

The antimicrobial activity of essential oils has been demonstrated by numerous researches. Thus, many antimicrobial methods have been suggested, most useful of which are the following:

- (i) The first method, reported by Tantaoui-Elaraki *et al.* (1992), is a combination of the Beylier-Maurel' technique (1976), and modified by Benjilali *et al.* (1986). The technique allows assessment of the Minimal Inhibitory Concentration (MIC) and the Minimal Lethal Concentration (MLC) of the oil. It consists of cultivating the micro-organisms on Millipore membranes of 0.45 µm porosity placed on agar media containing different concentrations of the tested oil.
- (ii) The second method is derived from the micro-atmosphere technique of Kellner and Kober, reported by Allegrini and Simeon de Buochberg (1972). This second technique consists of cultivating the micro-organisms to be tested in Petri dishes on agar medium and incubating these dishes in

reversed position after laying down the essential oil on a filter paper in the middle of the dish cover. The oil evaporates in the atmosphere of the dish and the volatile phase can exert its inhibitory effect on the inoculated micro-organisms.

- (iii) This latter technique was modified by Benjilali *et al.* (1984). The authors used Pyrex glass Petri dishes with exactly the same shape where the conidiospores were inoculated in radial lines. The Petri dishes were turned upside down and the filter paper was put in the middle of the cover and soaked with variable amounts of tested oil. For each 3–6 strains series, and for each oil, six Petri dishes were prepared. The partial inhibitory effect of essential oil was determined after incubation by comparison of the growth observed (length and width) to that of the control. This technique presents some advantage when compared to the original method of Kellner and Kober. First, it is inexpensive since it allows the study of several strains using the same Petri dish. Second, the radial inoculation offers the advantage of easily estimating the inhibition effect by measuring the extent of growth and thus, to compare several strains under the same conditions for their sensitivity.
 - (iv) Beylier-Maurel's method (1976) has also been modified by Benjilali *et al.* (1986) to study antimicrobial effects of other oils in a solid medium. This technique, unlike the preceding one, permitted testing per direct contact with the micro-organism, the totality of essential oil constituents. In addition, the minimum inhibitory concentration (MIC) is determined.
 - (v) Moreover, according to Santos *et al.* (1997), antibacterial screening of essential oils from *Psidium* and *Pilocarpus* species of plants was performed by agar dilution method (National Committee for Clinical Laboratory Standards, 1990) and the MIC was determined for each essential oil using a two-fold serial dilution technique (Hufford *et al.*, 1975). The essential oils were added at concentrations ranging from 50 to 3000 µg/ml to melted and cooled Muller Hinton agar and then distributed to sterile Petri dishes. The test bacterial organisms were inoculated in sterile saline (0.85%) on a 0.5 MacFarland standard (10^8 colony forming units (CFU)/ml) and this suspension was diluted 1:10 so as to obtain a final concentration of 10^7 CFU/ml. One micro-litre of this suspension was delivered to the agar surface resulting in the final desired inoculum of approximately 10^4 CFU per spot. The Petri dishes were then incubated at 37 °C for 18–20 h and after this period they were observed for the lowest concentration that inhibits visible growth which was recorded as the MIC.
- Therefore, it is important to mention the sensitivity or resistance of tested micro-organisms (Gram-positive and Gram-negative bacterium, yeast, mold or fungi) toward tested essential oils. In this case, the micro-organisms can be classified into three groups:
- (i) highly sensitive micro-organisms;
 - (ii) highly resistant micro-organisms, having a weak over-all sensitivity; and
 - (iii) micro-organisms with a medium over-all sensitivity.

Antiviral oils

Besides their antibacterial and antifungal properties, essential oils had also been reported to possess interesting antiviral activity. Bammi *et al.* (1997) demonstrated the effect of five essential oils on Epstein-Barr virus (EBV) (Viridae) which caused the infectious mononucleosis associated with Burkitt lymphoma and naso-pharynx carcinoma. The study aimed the effect of these oils on the expression of EBV viral capsid antigen (VCA) in the Marmouset B95-8 lymphoblastoid cellular line using the indirect immuno-fluorescence technique. The results showed a cytotoxic effect of tested oils at a dilution factor lower than 1:500. Moreover, the valour of cellular viability was not affected. Treatment of B95-8 cells with 1:1000 dilution of *Thymus* oil increased the fluorescence intensity of VCA-positive cells in two separate experiments. In three other tests, only fluorescence intensity was increased by oil from *Thymus* sp., while the percentage of the fluorescent cells did not increase significantly.

Insecticidal activity

Essential oils are also reported to have insecticide properties, essentially as ovicidal, larvicidal, growth inhibitor, repellency and antifeedant (Saxena and Koul, 1978; Dale and Saradamma, 1981; Schearer, 1984; Krishnarajah *et al.*, 1985; Nath *et al.*, 1986; Koul, 1987; Isman *et al.*, 1990; Shaaya *et al.*, 1991; Lahlou *et al.*, 2001d). The influence of certain oils and their constituents on the reproduction of some insect species and on morphological changes in others has also been discussed (Saxena and Rohdendory, 1974; Ramos and Stefen, 1986; Smet *et al.*, 1986).

Larvicidal and ovicidal oils/constituents. According to Laurent *et al.* (1997), 63 essential oils isolated from Bolivian plants were tested on *Triatoma infestans* for ovicidal and larvicidal properties. This insect is responsible for transmission of Chagas' disease to humans in the region extending from the arid Peruvian highlands to the very dry northeastern Brazilian regions, and the plains of Argentina. Three types of test were used: topical application on insects; nymphs on impregnated paper and eggs on impregnated paper. In all tests, the essential oils were used as ethanol solutions with concentrations of 2% and 20% (v:v).

- (i) For insects test, 1 μ l of each solution was applied directly over the abdomen of 10 fourth instar nymphs of *T. infestans*. After observing daily for a week, the nymphs were treated again with 5 μ l of the same solution. Two sets of controls were utilised. One control group was treated with ethanol only, while the other was not treated. The effect of the application was observed for another week and compared with the controls.
- (ii) For nymphs, 200 μ l of each ethanol solution of essential oils were deposited over filter paper disks which were dried at room temperature for 5 min and placed in Petri dishes, then 5 fourth instar nymphs of *T. infestans* were introduced in each dish. Insect control groups were treated in the same way but dosed only with ethanol. The effect knock-

down was observed daily for 4 days and compared with controls.

- (iii) For ovicidal tests, 10 eggs of *T. infestans* were introduced in each dish where the filter paper disks impregnated with a known volume of oil was deposited. The hatching of the larvae and the effect on them (knock-down or mortality) were observed every day until the control eggs completed hatching.

The results of this study indicated the effectiveness of 20 oils on nymphs and eggs when the impregnated paper tests were used. These tests proved to be the most sensitive and were therefore chosen for studying the action of 12 terpenes present in those active oils.

Growth inhibitors and antifeedants oils/constituents.

Asarones isolated from the essential oil of *Acorus calamus* L. rhizomes (Araceae) were potent growth inhibitors and antifeedants to the variegated cutworm, *Peridroma saucia* Hubner (Koul *et al.*, 1990). Experimental diets contained *cis*- or *trans*-asarone at concentrations of 60–2000 ppm fresh weight of diet. Larval weights and mortality were recorded after 7 and 10 days for first-instar larvae and after 2, 4, 6 and 8 days for third-instar larvae.

Nutritional experiments were carried out with fourth-instar larvae as these larger larvae and their frass could be more accurately weighed than that of the first three instars. In the first experiment, 20 larvae/concentration were provided with either compound at dietary concentrations of 250–1000 ppm. Relative growth per unit weight of the insect at the outset of the experiment (RGRi) and relative consumption per unit weight of the insect at the outset of the experiment (RCRi) were calculated on a dry weight basis after three days of feeding.

Cis- and *trans*-Asarone were also topically applied to fourth-instar larvae in 1 μ l acetone at doses of 5, 10, 20 and 30 μ g/larva, with appropriate controls treated with acetone alone. Twenty larvae were treated with each compound at each dose and were then allowed to feed on untreated diet for 72 h before insects, remaining food, and frass were dried at 60 °C to constant weight, and nutritional indices calculated.

Antifeedant activity was assayed using a leaf disk choice test. The 2.0-cm² disks were punched out from cabbage leaves and treated on each side with 15 μ l of aqueous asarone solutions emulsified with Triton X-100 (0.1%) to give concentrations ranging from 1.0 to 45.0 μ g/cm². Controls were treated with 15 μ l of the carrier alone. The leaf disks were dried at room temperature and then fourth- or fifth-instar larvae were introduced into each arena containing two treated and two control disks in alternate positions. Experiments were carried out with two larvae per Petri dish with five replicates for each treatment. Consumption was recorded using a digitising leaf area meter after 20 h for fourth-instar and 6 h for fifth-instar larvae.

Gross dietary utilisation (efficiency of conversion of ingested food, ECI) was decreased when the diet was supplemented with *cis*-asarone or when this compound was topically applied to fourth-instar larvae. Inhibition of growth occurred even at a moderate topical dose

(5 µl/larva) primarily as a result of decreased efficiency of conversion of digested food (ECD), even though the approximate digestibility (AD) of the food was unchanged. Oral or topical treatment with *trans*-asarone also significantly inhibited larval growth, but in this case the effect can be strictly attributed to decreased consumption, as dietary utilization (ECI) was not affected.

Antidiarrhoeal savoury

Hajhashemi *et al.* (2000) investigated essential oil from *Satureja hortensis* for treatment of some gastrointestinal problems. Isolated rat ileum was maintained in a water bath and spasmogenic agents, either potassium chloride (KCl) or acetylcholine (Ach) were added in varying concentrations. They caused a dose-dependent contraction of the tissues. The spasmolytic activities of dilutions of savoury essential oil were then determined by adding them to the water bath.

The oil was also investigated for *in-vivo* tests on mice. The test animals were orally given a 10% solution of savoury essential oil (1 mg/100 g) diluted in Tween 20, whilst the control animals were given either nothing or a solution of the emulsifier. Thirty minutes later, all of the mice were given castor oil in an attempt to induce diarrhoea but the animals in the essential oil group had no wet defecation.

The savoury essential oil was considered as spasmolytic towards the ileum by countering the effects of depolarisation caused by KCl and inhibiting the activation of muscarinic receptors by Ach.

Effects of essential oils/constituents on muscle: intestinal myorelaxant and antispasmodic effects

The effect of the essential oil of *Croton nepetaefolius* (EOCN), a medicinal plant from the north-east of Brazil, and its constituents cineole, methyl-eugenol and terpineol, were studied on intestinal motility *in-vivo* and on *in-vitro* mechanical activity of intestinal smooth muscle (Magalhães *et al.*, 1998).

In vivo experiments. Each experimental mouse received a quantity of charcoal 30 min after receiving EOCN or vehicle administered intragastrically. After a further 20 min the animals were killed and the small intestine removed. The distance travelled by the marker was measured and expressed as a percentage of the total intestinal length. In addition, some animals were used to determine the effect of EOCN on castor oil-induced alteration of intestinal transit.

In vitro experiments. Male guinea-pigs were killed and small segments of intestine and the gastro-oesophageal, pyloric and ileo-caecal sphincters were removed by careful dissection.

The tissues were mounted vertically in organ baths containing tyrode solution bubbled with air. In experiments with ileum, following an equilibration period of 1 h, two standard contractions were induced by adding 60 mM potassium chloride to the bath. The majority of the results presented were then normalised as a percentage of the mean of these initial contractions.

Concentration-effect curves for each agent tested were performed by cumulative additions to the bath in increasing concentration. In experiments examining the relaxation of the basal tonus of the ileum, paired segments of ileum were set up: one piece was exposed to the oil and the other received no treatment. Relaxation due to test substance was taken to be the difference between the tonus of control and test segment. Finally, in order to quantify the effect of EOCN on high K⁺-induced contractions, the preparations were exposed to the EOCN for a 5 min period after which the bath solution was changed for one containing 60 mM K⁺, still in the presence of EOCN.

Other methods investigated the effects of the essential oil of *Croton zehntneri* either on isolated smooth muscle preparations of the guinea-pig on skeletal muscles, in addition to anethole and estragole (Albuquerque *et al.*, 1995; Coelho-de-Souza *et al.*, 1998). The effects of essential oil of *Mentha x villosa* were also studied on skeletal muscle of the toad (Fogaça *et al.*, 1997) in addition to the effects of eugenol on excitation-contraction coupling in skeletal muscle (Leal-Cardoso *et al.*, 1994).

Antinociceptive effect of essential oil

The antinociceptive effect of leaf essential oil from *Psidium guajava* and its major constituents, β -caryophyllene and α -pinene was assessed by Santos *et al.* (1998) using chemical (formalin and acetic acid) and thermal (hot-plate) nociceptive tests in adult male albino mice.

Acetic acid-induced writhing test. The number of writhes per mouse were counted during a 10 min period, starting 10 min after *i.p.* administration of 0.6% acetic acid (10 ml/kg). Vehicle (2% Tween 80 in saline, 10 ml/kg), essential oil (100, 200 and 400 mg/kg) or its major components, β -caryophyllene (400 mg/kg) were administered orally 45 min before acetic acid injection.

Formalin test. Mice were injected with 20 µl of 1% formalin into the subplantar space of the right hind paw and the duration of paw licking was determined 0–5 min (first phase) and 20–25 min (second phase) after formalin. The *Psidium* essential oil suspended in 2% Tween 80 was administered orally at doses of 100, 200 and 400 mg/kg, 45 min before formalin injection at a dose of 10 ml/kg. Control animals received an equal volume of vehicle. To investigate the possible mechanisms involved in the antinociceptive effect of the essential oil, animals received caffeine (20 mg/kg, *i.p.*) or naloxone (1 mg/kg, *s.c.*) 15 min before oral administration of essential oil (400 mg/kg). The effect of β -caryophyllene and α -pinene, the two major components of plant oil were also tested at a dose of 400 mg/kg.

Hot-plate test. Mice were preselected by placing them individually on the hot-plate maintained at 51 ± 0.5 °C and animals that showed a reaction time greater than 20 s were discarded. The reaction time was measured before and 30, 60 and 90 min after oral administration of essential oil (100, 200 and 400 mg/kg), its major

components β -caryophyllene (400 mg/kg), α -pinene (400 mg/kg) or vehicle. A cut-off time of 45 s was used to prevent damage to the limbs.

Gastroprotective effect of essential oil constituent

According to Santos and Rao (2001), the gastroprotective effect of 1,8-cineole on ethanol-induced gastric mucosal damage in rats was performed as follow:

Gastric Mucosal Damage. Gastric mucosal damage was induced in conscious rats by intragastric instillation of ethanol. The test drug 1,8-cineole was administered at doses of 25, 50, 100 and 200 mg/kg body weight by oral gavage before ethanol. The animals were killed 60 min after ethanol and their stomachs removed and opened along the greater curvature to observe the lesions macroscopically. Lesion severity was determined by measuring the area of lesions with a transparent grid placed on the glandular mucosal surface.

Gastric Wall Mucus. The ethanol-induced changes on gastric mucus was indirectly analyzed by determining the amount of Alcian blue bound to the gastric wall. The mucus-dye complex was extracted by placing the segments in 10 ml of 0.5 M magnesium chloride for 2 h. Four milliliters of dye extract was mixed with an equal volume of diethyl ether. The quantity of Alcian blue extracted per gram of glandular tissue was then calculated.

Gastric Secretary Studies. The pylorus of each rat was ligated under light ether anesthesia to study the basal gastric secretion. Cineole and cimetidine were administered intraduodenally immediately after pylorus ligation. Control animals were given the vehicle instead of test drugs. The animals were killed after 4 h. The stomachs were removed and the volume of gastric juice was determined after centrifugation.

Gastric Mucosal Nonprotein Sulfhydryls (NP-SH). Gastric mucosal NP-SH were measured and the glandular stomachs from rats treated with 1,8-cineole or vehicle were removed and homogenised in ice-cold 0.02 M ethyl-enediaminetetraacetic acid. The homogenate was mixed and then centrifuged. The supernatants were mixed and the sample was shaken. The absorbance was then read.

Sedative effects of essential oils and fragrance compounds

In order to screen and quantify a series of fragrance compounds and essential oils for their sedative properties, groups of four mice each were exposed under standardised conditions to these compounds (Buchbauer *et al.*, 1993b). Thus, the motility of the animals was ascertained after inhalation and also after caffeine-induced overagitation to collect more detailed information on activating or sedating effects of several compounds and their aromatherapeutical usage. In

addition, serum samples of the mice were analyzed by gas chromatography-mass spectroscopy (GC-MS), gas chromatography-fourier transform infrared (GC-FTIR), and gas chromatography-flame ionization detection (GC-FID) to identify and quantify potent compounds effective in increasing or decreasing the motility of mice by inhalation alone. Fragrance compound motility data were correlated with data of the single-odor detection thresholds to compare volatility and motility effects.

CONCLUSION

It can be concluded that biological and pharmacological activities of essential oils must take into account different parameters and factors which can affect results of these studies (species, ecological factors and environmental conditions). Thus, each plant species presents a profile which it will express differently among these factors. For this reason, a few published studies on essential oils have aimed at elucidating the degree of specificity of the effect of these compounds among the various types of their bioactivity.

Moreover, the studies presented in this paper demonstrated the general utility of the bioassays using compounds of diverse structures and a complex chemical composition of such essential oils. The knowledge of the methods for testing essential oils/constituents is therefore indispensable in discovering the spectrum of action of these natural products, their modes of action and their therapeutic applications.

Nevertheless, it is worth noting that essential oils are very heterogeneous mixtures of single substances, biological actions are primarily due to these components in a very complicated concert of synergistic or antagonistic activities. Mixtures of such chemicals show a broad spectrum of biological effects and pharmacological properties. Moreover, these compounds seem to possess a distinct molecular formula, a certain molecular weight, and certain physicochemical properties.

Several factors such as phonological age of the plant, percent humidity of the harvested material, and the method of extraction have been identified as possible sources of variation for the chemical composition, toxicity and bioactivity of the extracts.

It is hoped that research institutes and universities will continue their efforts to discover other new natural active compounds derived from aromatic plants and develop their action spectrum on biological materials and pharmacological animal models to find other active natural molecules with potent therapeutic action devoid or less toxic than synthetic ones. It is also desirable that preclinical research on essential oils is a necessary part of the drug discovery and development process, especially given that there is no single forum and focus for guidance on determining the validity and relevance of preclinical data.

Finally, we should maintain our efforts in considering and valorising our natural patrimony, as well as conducting more scientific research on aromatic plants from chemical analysis, biological, toxicological and pharmacological investigations to therapeutic aspects.

REFERENCES

- Abbott WS. 1925. A method of computing the effectiveness of an insecticide. *J Econ Entomol* **18**: 265–267.
- Akinpelu DA, Olorunmola FO. 2000. Antimicrobial activity of *Bridelia ferruginea* fruit. *Fitoterapia* **71**: 75–76.
- Alaoui K, Lagorce J-F, Cherrah Y, et al. 1998. Activité analgésique et anti-inflammatoire des saponines d'*Argania spinosa*. *Ann Pharm Fr* **56**: 220–228.
- Albuquerque AAC, Sorenson AL, Leal-Cardoso JH. 1995. Effects of essential oil of *Croton zehntneri*, and of anethole and estragole on skeletal muscles. *J Ethnopharm* **49**: 41–49.
- Allegrini J, Simeon de Buochberg M. 1972. Une technique d'étude du pouvoir antibactérien des huiles essentielles. *Production et Problèmes Pharmaceutiques* **27**: 891–897.
- Allegrini J, Siméon de Buochberg M, Boillols H. 1973. Emulsions d'huiles essentielles-fabrication et application en microbiologie. *Travaux de la Société de Pharmacie de Montpellier* **33**: 73–86.
- Amos S, Binda L, Akah P, et al. 2003. Central inhibitory activity of the aqueous extract of *Crinum giganthum*. *Fitoterapia* **74**: 23–28.
- Asha KN, Chowdhury R, Hasan CM, Rashid MA. 2003. Antibacterial activity and cytotoxicity of extractives from *Uvaria hamiltonii* stem bark. *Fitoterapia* **74**: 159–163.
- Atindehou KK, Koné M, Tenneaux C, Traore D, Hostettman K, Dosso M. 2002. Evaluation of the antimicrobial potential of medicinal plants from the Ivory Coast. *Phytother Res* **16**: 497–502.
- Awad AHH, Probert AJ. 1991. The effect of praziquantel on the ultrastructure of *Schistosoma margrebowiei*. *J Helminth* **65**: 79–88.
- Aziba PI, Adedeji A, Ekor M, Adeyemi O. 2001. Analgesic activity of *Peperomia pellucida* aerial parts in mice. *Fitoterapia* **72**: 57–58.
- Baaliouamer A, Meklati B-Y, Fraisse D, Scharff C. 1992. The chemical composition of some cold-pressed Citrus oils produced in Algeria. *J Essent Oil Res* **4**: 251–258.
- Badilla B, Arias AY, Arias M, et al. 2003. Anti-inflammatory and antinociceptive activities of *Loasa speciosa* in rats and mice. *Fitoterapia* **74**: 45–51.
- Baggio CH, Freitas CS, Rieck L, Marques MCA. 2003. Gastroprotective effects of a crude extract of *Baccharis illinita* DC in rats. *Pharmacol Res* **47**: 93–98.
- Balansard G. 1990. Analyse critique des protocoles pharmacologiques utilisés pour la recherche d'extraits et de substances pures d'origine végétale à propriétés antibactérienne ou antiparasitaire. *Actes du 1er Colloque Européen d'Ethnopharmacologie Metz 22–25 mars*: 229–241.
- Bammi J, Khelifa R, Remmal A, et al. 1997. Etudes de l'activité antivirale de quelques huiles essentielles. In *Proceedings of the Intern. Congr. Arom. Medicinal Plants & Essential Oils*, Benjilali B, Ettalibi M, Ismaili-Alaoui M, Zrira S (eds). Actes Editions, Rabat, Morocco; 502.
- Bélangier A, Landry B, Dextra L, et al. 1991. Extraction et détermination de composés volatils de l'ail (*Allium sativum*). *Rivista Italiana EPPoS* **2**: 455.
- Benjilali B, Tantaoui-Elaraki A, Ayadi A, Ihlal M. 1984. Method to study antimicrobial effects of essentials: application to the antifungal activity of six Moroccan essences. *J Food Protection* **47**: 748–752.
- Benjilali B, Tantaoui-Elaraki A, Ismaili-Alaoui M, Ayadi A. 1986. Méthode d'étude des propriétés antiseptiques des huiles essentielles par contact direct en milieu gélosé. *Plantes Méd Phytother* **20**: 155–167.
- Beylier-Maurel MF. 1976. Activité bactériostatique des matières premières de parfumerie. *Rivista Italiana* **58**: 253–256.
- Beylier M. 1979. Bacteriostatic activity of some Australian essential oils. *Perfum Flavorist* **4**: 23–25.
- Biswas S, Murugesan T, Sinha S, et al. 2002. Antidiarrhoeal activity of *Strychnos potatorum* seed extract in rats. *Fitoterapia* **73**: 43–47.
- Bliss Cl. 1935. The calculation of the dosage mortality curve. *Ann Appl Biol* **22**: 134–167.
- Boskabady MH, Ramazani-Assari M. 2001. Relaxant effect of *Pimpinella anisum* on isolated guinea pig tracheal chains and its possible mechanism(s). *J Ethnopharm* **74**: 83–88.
- Boskabady MH, Khatami A. 2003. Relaxant effect of *Foeniculum vulgare* on isolated guinea pig tracheal chains. *Pharm Biol* **41**: 211–215.
- Boominathan R, Parimala Devi B, Mandal SC. 2003. Evaluation of antitussive potential of *Ionidium suffruticosam* Ging. (Violaceae) extract in albino mice. *Phytother Res* **17**: 838–839.
- Bouزيد N, Vilarem G, Gaset A. 1997. Extraction des huiles essentielles par des technologies non conventionnelles. In *Proceedings of the Intern. Congr. Arom. Medicinal Plants & Essential Oils*, Benjilali B, Ettalibi M, Ismaili-Alaoui M, Zrira S (eds). Actes Editions, Rabat, Morocco; 115–120.
- Brosseau M. 1997. Procédé assisté par micro-onde (MAP™): application à l'extraction d'huiles essentielles. In *Proceedings of the Intern. Congr. Arom. Medicinal Plants & Essential Oils*, Benjilali B, Ettalibi M, Ismaili-Alaoui M, Zrira S (eds). Actes Editions, Rabat, Morocco; 121–126.
- Bruneton J. 1995. *Pharmacognosy, Phytochemistry, Medicinal Plants*. Intercept, Ltd.: Hampshire.
- Buchbauer G, Jager W, Jirovetz L, Imberger J, Dietrich H. 1993a. Therapeutic properties of essential oils and fragrances. In *Bioactive volatile compounds from plants*, Teranishi R, Buttery R, Sugisawa H (eds); 161.
- Buchbauer G, Jirovetz L, Jager W, et al. 1993b. Fragrance compounds and essential oils with sedative effects upon inhalation. *J Pharm Sc*: **82**: 660–664.
- Buchbauer G. 2000. The detailed analysis of essential oils leads to the understanding of their properties. *Perfumer & Flavorist* **25**: 64–67.
- Chakrabarti S, Biswas TK, Rokeya B, et al. 2003. Advanced studies on the hypoglycemic effect of *Caesalpinia bonducella* F. in type 1 and 2 diabetes in Long Evans rats. *J Ethnopharm* **84**: 41–46.
- Chalchat JC, Garry RP, Bastide P, et al. 1991. Corrélation composition chimique/activité antimicrobienne: V- Contribution à la comparaison de 2 méthodes de détermination des CMI. *Plantes Méd Phytother* **XXV**: 184–193.
- Chanegriha N, Sabaou N, Baaliouamer A, Meklati BY. 1994. Activité antibactérienne et antifongique de l'huile essentielle du Cyprés d'Algérie. *Rivista Italiana*: **12**: 5–10.
- Chattopadhyay RR, Medda C, Das S, Basu TK. 1993. Hypoglycemic and antihyperglycemic effect of *Gymnema sylvestre* leaf extract in rats. *Fitoterapia* **LXIV**: 450–454.
- Chaves MC, Santos FA, Meriezes AMS, Rao VSN. 1998. Experimental evaluation of *Myracrodruon urundeuva* bark extract for antidiarrhoeal activity. *Phytother Res* **12**: 549–552.
- Chiasson H, Bélangier A, Bostanian N, et al. 2001. Acaricidal properties of *Artemisia absinthium* and *Tanacetum vulgare* (Asteraceae) essential oils obtained by three methods of extraction. *J Econ Entomol* **94**: 167–171.
- Choi H-S, Kim H-S, Min KR, et al. 2000. Anti-inflammatory effects of fangchinoline and tetrandrine. *J Ethnopharm* **69**: 173–179.
- Choi S, Jung S-Y, Kim C-H, et al. 2001. Effect of ginsenosides on voltage-dependent Ca²⁺ channel subtypes in bovine chromaffin cells. *J Ethnopharm* **74**: 75–81.
- Chowdhury D, Sayeed A, Islam A, et al. 2002. Antimicrobial activity and cytotoxicity of *Aerva lanata*. *Fitoterapia* **73**: 92–94.
- Chowdhury R, Hasan CM, Rashid MA. 2003. Antimicrobial activity of *Toona ciliata* and *Amoora rohituka*. *Fitoterapia* **74**: 155–158.
- Coelho-de-Souza AN, Criddle DN, Leal-Cardoso JH. 1998. Selective modulatory effects of the essential oil of *Croton zehntneri* on isolated smooth muscle preparations of the guinea pig. *Phytother Res* **12**: 189–194.
- Collin GJ, Lord D, Allaire J, Gagnon D. 1991. Huiles essentielles et extraits 'micro-ondes'. *Parfums Cosmétiques Arômes* **97**: 105–112.
- Conner DE, Beuchat LR. 1984. Sensitivity of heat-stressed yeast's to essential oils of plants. *Applied Environment Microbiol* **47**: 229–233.
- Copland A, Nahar L, Tomlinson CTM, et al. 2003. Antibacterial and free radical scavenging activity of the seeds of *Agrimonia eupatoria*. *Fitoterapia* **74**: 133–135.
- Dale D, Saradamma K. 1981. Insect antifeedant action of some essential oils. *Pesticides* **15**: 21.

- Dayal B, Purohit RM. 1971. Screening of some Indian essential oils for their antifungal properties. *The Flavour Industry* **2**: 484–485.
- Dellacassa E, Rossini C, Menendez P, *et al.* 1992. Citrus essential oils of Uruguay. Part I. Composition of oils of some varieties of Mandarin. *J Essent Oil Res* **4**, 265–272.
- Denayer R, Tilquin B. 1994. Détermination des indices de rétention de composants d'huiles essentielles. *Rivista Italiana* **13**: 7–12.
- Dongmo AB, Kamanyi A, Dzikouk G, *et al.* 2003. Anti-inflammatory and analgesic properties of the stem bark extract of *Mitragyna ciliata* (Rubiaceae) Aubrév. & Pellegr. *J Ethnopharm* **84**: 17–21.
- Dupont C. 1970. Détermination de la DL50 chez la souris: Méthode de Litchfield et Wilcoxon. *J Pharmacol* **1**: 407–414.
- Ebi GC. 2001. Antimicrobial activity of *Alchornea cordifolia*. *Fitoterapia* **72**: 69–72.
- El Mahi M, Essassi EM, Hmamouchi M, Hamamouchi J. 1997. Etude de l'activité antimicrobienne et antibilharzienne de *Zizyphus vulgaris*. *Fitoterapia* **LXVIII**: 284–286.
- Erdemoglu N, Sener B. 2001. Antimicrobial activity of the heartwood of *Taxus baccata*. *Fitoterapia* **72**: 59–61.
- Finney DJ. 1971. *Probit analysis*, 3rd edn. Cambridge University Press: Cambridge.
- Fleischer TC, Ameade EPK, Sawyer IK. 2003a. Antimicrobial activity of the leaves and flowering tops of *Acanthospermum hispidum*. *Fitoterapia* **74**: 130–132.
- Fleischer TC, Ameade EPK, Mensah MLK, Sawyer IK. 2003b. Antimicrobial activity of the leaves and seeds of *Bixa orellana*. *Fitoterapia* **74**: 136–138.
- Fogaça RTH, Cavalcante ADA, Serpa AKL, *et al.* 1997. The effects of essential oil of *Mentha x villosa* on skeletal muscle of the toad. *Phytother Res* **11**: 552–557.
- Franchomme P. 1981. L'anomatologie à visée anti-infectieuse. *Phytopharmacie* **1–2**: 25–47.
- Gebremedhin G, Adewunmi CO, Becker W, *et al.* 1994. Hirudinicidal activities of some natural molluscicides used in schistosomiasis control. *J Ethnopharm* **41**: 127–132.
- Ghoulami S, Oumzil H, Rhajaoui M, *et al.* 2001. *Essais de valorisation du genre Mentha x villosa du Maroc. Extraction au micro-onde et activité antimicrobienne. Premier Congrès de l'AMPEPM: Plantes Médicinales. Biologie et Santé: Kenitra, Morocco*; 49.
- Gokhale AB, Damre AS, Saraf MN. 2003. Investigations into the immunomodulatory activity of *Argyrea speciosa*. *J Ethnopharm* **84**: 109–114.
- Guide for the Care and Use of Laboratory Animals. 1985. US Department of Health and Human Services, Public Health Service, National Institutes of Health, NIH Publication No. 86–23, Revised 1985 or Succeeding Revised Editions.
- Gupta M, Mazumder UK, Manikandan L, *et al.* 2003. Antibacterial activity of *Vernonia cinerea*. *Fitoterapia* **74**: 148–150.
- Gupta M, Mazumder UK, Manikandan L, *et al.* 2003. Evaluation of antipyretic potential of *Vernonia cinerea* extracts in rats. *Phytother Res* **17**: 804–806.
- Hajhashemi V, Sadraei H, Ghannadi AR, Mohseni M. 2000. Antispasmodic and anti-diarrhoeal effect of *Satureja hortensis* L. essential oils. *J Ethnopharmacol* **71**: 187–192.
- Hmamouchi M, Lahlou M, Essafi N, Agoumi A. 1998. Molluscicidal properties of some proanthocyanidins, flavones and flavonols. *Fitoterapia* **19**: 161–164.
- Hmamouchi M, Lahlou M, Agoumi A. 2000. Molluscicidal activity of some Moroccan medicinal plants. *Fitoterapia* **71**: 308–314.
- Hufford CD, Funderburk MJ, Morgan JM, Robertson LW. 1975. Two antimicrobial alkaloids from heartwood of *Liriodendron tulipifera* L. *J Pharmac Sci* **64**: 788–792.
- Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Testing and Education. 1988. Ad Hoc Animal research Committee, New York Academy of Sciences, 1988.
- International Guiding Principles for Biomedical Research Involving Animals. 1985. Council for International Organisations of Medical Sciences (CIOMS), Geneva, 1985.
- Isah AB, Ibrahim YKE, Iwalewa EO. 2003. Evaluation of the antimalarial properties and standardization of tablets of *Azadirachta indica* (Meliaceae) in mice. *Phytother Res* **17**: 807–810.
- Ishih A, Ikeya C, Yanoh M, *et al.* 2001. A potent antimalarial activity of *Hydrangea macrophylla* var. *Otaksa* leaf extract against *Plasmodium yoelii* 17XL in mice. *Parasit Inter* **50**: 33–39.
- Isman MB, Koul O, Luczynski A, Kaminski J. 1990. Insecticidal and antifeedant bioactivities of Neem oils and their relationship to Azadirachtin content. *J Agric Food Chem* **38**: 1406.
- Janssen AM, Scheffer JJ, Baerheim Svendsen A. 1987. Antimicrobial activity of essential oils: a 1976–1986 literature review. Aspects of the test methods. *Planta Med* **53**: 395–398.
- Jantan I, Zaki ZM, Ahmad AR, Ahmad R. 1999. Evaluation of smoke from mosquito coils containing Malaysian plants against *Aedes aegypti*. *Fitoterapia* **70**: 237–243.
- Kameswara RB, Giri R, Kesavulu MM, Apparao C. 2001. Effect of oral administration of bark extracts of *Pterocarpus santalinus* L. on blood glucose level in experimental animals. *J Ethnopharm* **74**: 69–74.
- Kariba RM, Siboe GM, Dossaji SF. 2001. *In vitro* antifungal activity of *Schizozygia coffaeoides* Bail. (Apocynaceae) extracts. *J Ethnopharm* **74**: 41–44.
- Keita A, Doumbo O, Koita N, *et al.* 1990. Recherche expérimentale sur un anti-malarique traditionnel. *Bull Méd Trad Pharm* **4**: 139–145.
- Keravis G. 1997. Spectrométrie de masse et chromatographie dans l'analyse des plantes aromatiques et huiles essentielles. In *Proceedings of the Intern. Congr. Arom. Medicinal Plants & Essential Oils*, Benjilali B, Ettalibi M, Ismaili-Alaoui M, Zrira S. (eds). Actes Editions, Rabat, Morocco; 379–384.
- Khan MR, Kihara M, Omoloso AD. 2000. Antimicrobial activity of *Evodia elleryana*. *Fitoterapia* **71**: 72–74.
- Koul O. 1987. Antifeedant and growth inhibitory effects of Calamus oils and Neem oil on *Spodoptera litura* under laboratory conditions. *Phytoparasitica* **15**: 169.
- Koul O, Smirle MJ, Isman MB. 1990. Asarones *Acorus calamus* L. oil: their effect on feeding behaviour and dietary utilisation in *Peridroma saucia*. *J Chem Ecol* **16**: 1911–1920.
- Krishnarajah SR, Ganesalingam VK, Senanayake UM. 1985. Repellency and toxicity of some plant oils and their terpene components to *Sitotraga cerealilla* (Olivier, Lepidoptera, Gelechiidae). *Trop Sci* **25**: 249.
- Kumarasamy Y, Nahar L, Sarker SD. 2003a. Bioactivity of gentiopicoside from the aerial parts of *Centaureum erythraea*. *Fitoterapia* **74**: 151–154.
- Kumarasamy Y, Nahar L, Cox PJ, *et al.* 2003b. Biological activity of lignans from the seeds of *Centaurea scabiosa*. *Pharm Biol* **41**: 203–206.
- Lacoste E, Chaumont JP, Mandin D, *et al.* 1996. Les propriétés antiseptiques de l'huile essentielle de *Lippia sidoides* Cham. Application à la microflore cutanée. *Ann Pharm Fr* **54**: 228–230.
- Lahlou S, Leal-Cardoso JH, Magalhães PJC, *et al.* 1999. Cardiovascular effects of the essential oil of *Croton nepetaefolius* in rats: Role of the autonomic nervous system. *Planta Med* **65**: 553–557.
- Lahlou S, Leal-Cardoso JH, Magalhães PJC. 2000a. Essential oil of *Croton nepetaefolius* decreases blood pressure through an action upon vascular smooth muscle: Studies in DOCA-Salt hypertensive rats. *Planta Med* **66**: 138–143.
- Lahlou M, Berrada R, Agoumi A, Hmamouchi M. 2000b. The potential effectiveness of essential oils in the control of human head lice in Morocco. *Int J Aromather* **10**: 108–123.
- Lahlou M. 2001. Contribution à l'étude des activités antiparasitaires et insecticides par les plantes médicinales Marocaines. Doctoral Thesis, Faculty of Sciences Ain Chock, Casablanca, 300 pp.
- Lahlou M, Berrada R, Hmamouchi M. 2001a. Molluscicidal activity of thirty essential oils on *Bulinus truncatus*. *Thérapie* **56**: 71–72.
- Lahlou M, Berrada R. 2001b. Potential of essential oils in schistosomiasis control in Morocco. *Int J Aromather* **11**: 87–96.
- Lahlou M, Berrada R. 2001c. Cercaricidal activity of three Moroccan medicinal plants. *Thérapie* **56**: 441–442.
- Lahlou M, Berrada R, Hmamouchi M, Lyagoubi M. 2001d. Effect of some Moroccan medicinal plants on mosquito larvae. *Thérapie* **56**: 193–196.
- Lahlou M. 2002. Potential of *Origanum compactum* as a cercaricide in Morocco. *Annals Trop Med Parasit* **96**: 587–593.

- Lahlou M, El Mahi M, Hamamouchi J. 2002. Evaluation des activités antifongique et molluscicide de *Zizyphus lotus* (L.) Desf. du Maroc. *Ann Pharm Fr* **60**: 410–414.
- Lahlou M. 2003. Composition and molluscicidal properties of essential oils of five Moroccan Pinaceae. *Pharm Biol* **41**, 207–210.
- Lahlou M, Berrada R. 2003. Composition and niticidal activity of essential oils of three chemotypes of *Rosmarinus officinalis* L. acclimatised in Morocco. *Flav Frag J* **18**: 124–127.
- Lahlou M, Weliou M, Salem M, Hajji MS. 2003. Synthèse et activité insecticide de quelques dérivés benzimidazoliques et benzothiazoliques. *Ann Pharm Fr* **61**: 57–61.
- Lamchouri F, Settaf A, Cherrah Y, et al. 1999. Antitumour principles from *Peganum harmala* seeds. *Thérapie* **54**: 753–758.
- Larhsini M, Oumouli L, Lazrek HB, et al. 1999. Screening of antibacterial and antiparasitic activities of six Moroccan medicinal plants. *Thérapie* **54**: 763–765.
- Lattaoui N, Tantaoui-Elaraki A. 1994. Individual and combined antibacterial activity of the main components of three thyme essential oils. *Rivista Italiana* **13**: 13–19.
- Lattaoui N, Tantaoui-Elaraki A. 1997. Activité antimicrobienne des huiles essentielles de plantes aromatiques sur des bactéries d'intérêt hygiénique dans l'alimentation. In *Proceedings of the Intern. Congr. Arom. Med. Plants & Ess. Oils*, Benjilali B, Ettalibi M, Ismaili-Alaoui M, Zrira S. (eds). Actes Editions, Rabat, Morocco; 321–326.
- Laurent D, Vilaseca LA, Chantraine JM, et al. 1997. Insecticidal activity of essential oils on *Triatoma infestans*. *Phytother Res* **11**: 285–290.
- Lawrence BM. 2000. Essential oils: from agriculture to chemistry. *Int J Aromather* **10**: 82–98.
- Leal-Cardoso JH, Coelho-de-Souza AN, Souza IT, Figueiredo IMB. 1994. Effects of eugenol on excitation-contraction coupling in skeletal muscle. *Arch Inst Pharmacod Thérapie* **327**: 113–123.
- Lens-Lisbonne C, Cremieux A, Maillard C, Balansard D. 1987. Méthodes d'évaluation de l'activité antibactérienne des huiles essentielles: Application aux essences de Thym et de Cannelle. *J Pharm Belg* **42**: 297–302.
- Magalhães PJC, Criddle DN, Raquel AT, et al. 1998. Intestinal myorelaxant and anti-spasmodic effects of the essential oil of *Croton nepetaefolius*, and its constituents cineole, methyl-eugenol and terpineol. *Phytother Res* **12**: 172–177.
- Mandal SC, Lakshmi SM, Kumar CKA, et al. 2003. Evaluation of anti-inflammatory potential of *Pavetta indica* Linn. Leaf extract (Family: Rubiaceae) in rats. *Phytother Res* **17**: 817–820.
- Marquina S, Maldonado N, Garduno-Ramirez ML, et al. 2001. Bioactive oleanolic acid saponins and other constituents from the roots of *Viguiera decurrens*. *Phytochem* **56**: 93–97.
- Martin T, Villaescusa L, Diaz AM, et al. 1997. Screening for antiparasitic activity of *Myrtus communis*. *Fitoterapia* **LXVIII**: 267–277.
- Martinez Nadal NG, Montalvo AE, Seda M. 1973. Antimicrobial properties of bay and other phenolic essential oils. *Cosmetics Perfumery* **83**: 37–38.
- Medhi B, Khanikar HN, Lahon LC, et al. 2003. Analgesic, anti-inflammatory and local anaesthetic activity of *Moringa pterygosperma* in laboratory animals. *Pharm Biol* **41**: 248–252.
- Mikus J, Harkenthal M, Steverding D, Reichling J. 2000. *In vitro* effect of essential oils and isolated mono- and sesquiterpenes on *Leishmania major* and *Trypanosoma brucei*. *Planta Med* **66**: 366–368.
- Morris JA, Khetty A, Seitz EW. 1978. Antimicrobial activity of aroma and essential oils. *J Amer Oils Chem Soc* **56**: 595–603.
- Muko KN, Ohiri FC. 2000. A preliminary study on the anti-inflammatory properties of *Emilia sonchifolia* leaf extracts. *Fitoterapia* **71**: 65–68.
- Munoz V, Sauvain M, Bourdy G, et al. 2000. A search for natural bioactive compounds in Bolivia through a multidisciplinary approach Part I. Evaluation of the antimalarial activity of plants used by the Chacobo Indians. *J Ethnopharm* **69**: 127–137.
- Nath DR, Das NG, Malhotra PR. 1986. Efficacy of certain essential oils as insects repellents. *Def Sci J* **36**: 327.
- National Committee for Clinical Laboratory Standards. 1990. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically. Approved Standard M₇-A₂. National Committee for Clinical Laboratory Standards, Villanova, PA.
- Ndamba J, Nyazema N, Makaza N, et al. 1994. Traditional herbal remedies used for the treatment of urinary schistosomiasis in Zimbabwe. *J Ethnopharm* **42**: 125–132.
- Ogunwande IA, Bello MO, Olawore ON, Muili KA. 2001. Phytochemical and antimicrobial studies on *Butyrospermum paradoxum*. *Fitoterapia* **72**: 54–56.
- Ohiri FC, Esimone CO, Nwafor SV, et al. 2003. Hypoglycemic properties of *Viscum album* (Mistletoe) in alloxan-induced diabetic animals. *Pharm Biol* **41**: 184–187.
- Omer MEFA, Elnima EI. 2003. Antimicrobial activity of *Ximenia americana*. *Fitoterapia* **74**: 122–126.
- Onyeyili PA, Nwosu CO, Amin JD, Jibike JI. 2001. Anthelmintic activity of crude aqueous extract of *Nauclea latifolia* stem bark against ovine nematodes. *Fitoterapia* **72**: 12–21.
- Panagouleas C, Skaltsa H, Laazari D, et al. 2003. Antifungal activity of secondary metabolites of *Centaurea raphanina* ssp. *mixta*, growing wild in Greece. *Pharm Biol* **41**: 266–270.
- Panizzi L, Flamini G, Cioni PL, Morelli I. 1993. Composition and antimicrobial properties of essential oils of four Mediterranean Lamiaceae. *J Ethnopharmacol* **39**: 167–170.
- Paré JR, Sigouin M, Lapointe J. 1989. Microwave assisted natural product extraction. *Brevet App. Can.* No. 600322, 16 Mai.
- Pellecuer J, Allegrini J, Simeon-de-Buochberg M. 1976. Huiles essentielles bactéricides et fongicides. *Rev Ins Pasteur* (Lyon) **9**: 135–159.
- Perez C, Tiraboschi IN, Ortega MG, et al. 2003. Further antimicrobial studies of 2'-4'-dihydroxy-5'-(1'-dimethylallyl)-6-prenylpinocembrin from *Dalea elegans*. *Pharm Biol* **41**: 171–174.
- Rahman MT, Ahmed M, Alimuzzaman M, Shilpi JA. 2003. Antinociceptive activity of the aerial parts of *Solanum xanthocarpum*. *Fitoterapia* **74**: 119–121.
- Rahalison L, Hamburger M, Monod M, et al. 1994. Antifungal tests in phytochemical investigations: Comparison of bioautographic methods using phytopathogenic and human pathogenic fungi. *Planta Med* **60**: 41–44.
- Rakotonirina VS, Bum EN, Rakotonirina A, Bopelet M. 2001. Sedative properties of the decoction of the rhizome of *Cyperus articulatus*. *Fitoterapia* **72**: 22–29.
- Ramos O, Stefen H. 1986. The influence of Calamus oil and asarone analogues on the reproduction of *Oncopeltus fasciatus* (Dallas). *Philipp Entomol* **6**: 495.
- Reed LJ, Muench H. 1938. A simple method of estimating 50% endpoints. *The Am J Hygiene* **27**: 493–497.
- Remmal A, Bouchikhi T, Rhayour K, et al. 1993a. Improved method for the determination of antimicrobial activity of essential oils in agar medium. *J Ess Oil Res* **5**: 179–184.
- Remmal A, Bouchikhi T, Tantaoui-Elaraki A, Ettayebi M. 1993b. Inhibition of antibacterial activity of essential oils by Tween 80 and ethanol in liquid medium. *J Pharm Belg* **48**: 352–356.
- Remmal A. 1994. Activités antibactériennes et antivirales des huiles essentielles d'Origan, de Girofle et de Thym. Doctoral Thesis, Faculty of Sciences Dhar El Mehraz, Fés, Morocco.
- Remmal A, Rhayour K, Bouchikhi T, Tantaoui-Elaraki A. 2001. *Dispersion des huiles essentielles en milieu aqueux sans détergent: application à l'activité antibactérienne*. Premier Congrès de l'AMPEPM: Plantes Médicinales: Biologie et Santé. Kenitra: Morocco; 60.
- Rhiouani H, Settaf A, Lyoussi B, et al. 1999. Effects of saponins from *Herniaria glabra* on blood pressure and renal function in spontaneously hypertensive rats. *Thérapie* **54**: 735–739.
- Rojas G, Lévaro J, Tortoriello J, Navarro V. 2001. Antimicrobial evaluation of certain plants used in Mexican traditional medicine for the treatment of respiratory diseases. *J Ethnopharmacol* **74**: 97–101.
- Rub Nawaz H, Malik A, Shaiq Ali M. 2001. Trianthemol: an antifungal tetraterpenoid from *Trianthema portulacastrum* (Aizoaceae). *Phytochemistry* **56**: 99–102.
- Safir O, Fkih-Tetouani S, Soufiaoui M, et al. 1998. Microwave extraction of the aerial parts of *Zygophyllum gaetulum*. *Rivista Italiana* **25**: 3–10.
- Sairam K, Hemalatha S, Kumar A, et al. 2003. Evaluation of anti-diarrhoeal activity in seed extracts of *Mangifera indica*. *J Ethnopharm* **84**: 11–15.

- Santos FA, Cunha GMA, Viana GSB, *et al.* 1997. Antibacterial activity of essential oils from *Psidium* and *Pilocarpus* species of plants. *Phytother Res* **11**: 67–69.
- Santos FA, Rao VS, Silveira ER. 1998. Investigations on the antinociceptive effect of *Psidium guajava* leaf essential oil and its major constituents. *Phytother Res* **12**: 24–27.
- Santos FA, Rao VS. 2001. 1,8-cineole, a food flavoring agent prevents ethanol-induced gastric injury in rats. *Digestive Dis Sci* **46**: 331–337.
- Saxena BP, Rohdendory EB. 1974. Morphological changes in *Thermobia domestica* under the influence of *Acorus calamus* oil vapours. *Experientia* **30**: 1298–1300.
- Saxena BP, Koul O. 1978. Utilisation of essential oils for insect control. *Indian Perfum* **22**: 139.
- Sayyah M, Saroukhani G, Peirovi A, Kamalinejad M. 2003. Analgesic and anti-inflammatory activity of the leaf essential oil of *Laurus nobilis* Linn. *Phytother Res* **17**: 733–736.
- Scheerer WR. 1984. Components of oil of Tansy (*Tanacetum vulgare*) that repel Colorado potato beetles (*Leptinotarsa decemlineata*). *J Nat Prod* **47**: 964.
- Schlemper SR de M, Schlemper V, da Silva D, *et al.* 2001. Antibacterial activity of *Persea cordata* stem barks. *Fitoterapia* **72**: 73–75.
- Schofield CJ. 1994. Triatominae: biology and control. Euro-communic Publications: Bognst Regis, UK.
- Shaaya E, Ravid U, Paster N, *et al.* 1991. Fumigant toxicity of essential oils against four major stored-product insects. *J Chem Ecol* **17**: 499.
- Shuhua X, Jiqing Y, Jinying M, *et al.* 2000. Effect of praziquantel together with artemether on *Schistosoma japonicum* parasites of different ages in rabbits. *Parasit Inter* **49**: 25–30.
- Simeon de Buochberg M. 1976. Etude de l'activité antimicrobienne de l'huile essentielle de *Thymus vulgaris* L. et de ses constituants. Doctoral Thesis, Montpellier, France.
- Skim F, Lazrek HB, Kaaya A, *et al.* 1999. Pharmacological studies of two antidiabetic plants: *Globularia alypum* and *Zygophyllum gaetulum*. *Thérapie* **54**: 711–715.
- Smet H, van Mellaert H, Rans M, Loof A. 1986. The effect on mortality and reproduction of β -asarone vapors on two insect species of stored grain: *Ephestia kuehniella* (Lepidoptera) and *Tribolium confusum* Duval (Coleoptera). *Med Fac Landbonwet Rijksuniv Gent* **51**: 1197.
- Somchit MN, Reezal I, Elysha Nur I, Mutalib AR. 2003. *In vitro* antimicrobial activity of ethanol and water extracts of *Cassia alata*. *J Ethnopharmacol* **84**: 1–4.
- Susplugas P, Mongold JJ, Taillade C, Serrano JJ. 1993. Anti-inflammatory and analgesic activity of *Reseda phyteuma*. *Planters Méd Phytothér* **XXVI**: 375–382.
- Swaroop S. 1966. *Statistical Methods in Malaria Eradication*, WHO: Geneva.
- Takaisi-Kikuni NB, Tshilanda D, Babady B. 2000. Antibacterial activity of the essential oil of *Cymbopogon densiflorus*. *Fitoterapia* **71**: 69–71.
- Tantaoui-Elaraki A, Errifi A, Benjilali B, Lattaoui N. 1992. Antimicrobial activity of four chemically different essential oils. *Rivista Italiana* **6**: 13–22.
- Tantaoui-Elaraki A, Lattaoui N, Errifi A, Benjilali B. 1993. Composition and antimicrobial activity of the essential oils of *Thymus broussonettii*, *T. zygis* and *T. Satureioides*. *J Ess Oil Res* **5**: 45–53.
- Tantaoui-Elaraki A. 1997. Pouvoir antimicrobien des huiles essentielles des plantes aromatiques. In *Proceedings of the Intern. Congr. Arom. Med. Plants & Ess. Oils*, Benjilali B, Ettalibi M, Ismaili-Alaoui M, Zrira S (eds). Actes Editions, Rabat, Morocco; 301–310.
- Tapia-Pérez ME, Tapia-Contreras A, Cedillo-Rivera R, *et al.* 2003. Screening of Mexican medicinal plants for antiprotozoal activity – Part II. *Pharm Biol* **41**: 180–183.
- Teixeira MJ, de Almeida YM, Viana JR, *et al.* 2001. *In vitro* and *in vivo* leishmanicidal activity of 2-Hydroxy-3-(3-methyl-2-butenyl)-1,4-naphthoquinone (Lapachol). *Phytother Res* **15**: 44–48.
- Testa J, Kaimba C, Delmont J. 1991. Traitement traditionnel des teignes en RCA. *Revue Méd Pharm Afr* **5**: 51–55.
- Tomi F, Bighelli A, Bradesi P, Casanova J. 1997. Analyse des huiles essentielles par RMN ¹³C. In *Proceedings of the Intern. Congr. Arom. Med. Plants & Ess. Oils*, Benjilali B, Ettalibi M, Ismaili-Alaoui M, Zrira S (eds). Actes Editions, Rabat, Morocco; 499.
- Use of Laboratory Animals in Biomedical and Behavioural Research. 1988. Committee on the Use of Laboratory Animals in Biomedical and Behavioural Research, Commission on Life Sciences, National Research Council, Institute of Medicine, National Academy Press, Washington, DC.
- Vollekov A, Kostalova D, Kettmann V, Tth J. 2003. Antifungal activity of *Mahonia aquifolium* extract and its major protoberberine alkaloids. *Phytother Res* **17**: 834–837.
- WHO. 1970. Meeting of directors of collaborating laboratories on molluscicide testing and evaluation. *WHO/SCHISTO/71.6*: 1–33.
- WHO. 1981. Instructions for determining the susceptibility or resistance of mosquito larvae to insecticides. *WHO/VBC/81.807*: 1–6.