Chapter 16
Phytochemistry and Biotechnology Approaches of the Genus *Exacum*

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Abstract The genus *Exacum* consists of about 70 species occurring in the region of the Indian Ocean (Africa, Madagascar, Socotra, Arabian Peninsula, Sri Lanka, and India) and also in the Himalayas, southern Asia (China, Malaysia), and northern Australia. Until now, only the species *Exacum affine* has been cultured as an ornamental pot plant, but several other species also have features desired by horticulturists. Biotechnological methods for plant multiplication can be helpful to introduce plants into commercial floriculture. The genus *Exacum* is poorly studied in terms of the content of its chemical compounds. Major uses in traditional medicine, confirmed by ethnobotanical studies and investigations on biological activities, suggest great pharmacological potential of *Exacum* species. Plants derived from cultured tissues could be a source of material for the isolation of pharmaceutically important compounds. The accumulation of secondary metabolites in such cultures may be improved and modified using biotechnological approaches. Numerous *Exacum* species are endemic and often endangered by over-exploitation for medicinal purposes. Micropropagation methods can have application in the protection of those species.

16.1 Introduction

The genus *Exacum* is extremely interesting as a potential source of plant-based medicines and ornamentals but is poorly studied.

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16.2 Characteristics of the Genus *Exacum*

16.2.1 Occurence and Taxonomy

The genus *Exacum* is one of six genera belonging to the subfamily *Exaceae* and, according to the latest data (Wohlhauser and Callmander 2012), consists of about 70 species. In his monograph, Klackenberg (1985) described 65 species. They are found in the region of the Indian Ocean, in Africa, in Madagascar, on the island of Socotra, on the Arabian Peninsula, in Sri Lanka, India, the Himalayas, southern Asia (China, Malaysia), and in northern Australia. Research on the origin of this genus has shown that it comes from the island of Madagascar, where most of its species (39 species) can be found. Second in terms of the number of *Exacum* species is the India–Sri Lanka region (18 species). Klackenberg (1985) divides the genus *Exacum* into two sections: Section *Africana*, which includes species from Madagascar, Africa, the island of Socotra, and southern parts of the Arabian peninsula; and Section *Exacum*, which includes species from other regions, such as Sri Lanka and India (Struwe et al. 2002). Studies on the phylogeny and the biogeography of *Exacum* (*Gentianaceae*) showed a disjunctive distribution in the Indian Ocean basin resulting from long-distance dispersal and extensive radiation (Yuan et al. 2005).

Many species are endemic or rare and little known, but there are also a number of well-known and widespread species in natural environments, such as *E. tetragonum* (India, southern Asia, Australia), *E. pedunculatum* (India, Sri Lanka), *E. quinquenervium* (Madagascar, Mauritius), and *E. oldenlandoides* (tropical Africa). Plants of the genus *Exacum* grow in different natural conditions, from sea level to an altitude of 2800 m in Madagascar, or 2000 m above sea level in the Himalayas, in southern India and New Guinea. They grow in lowlands, meadows, marshes, and rocky areas (Struwe et al. 2002).

The genus *Exacum* was discovered by Linnaeus in 1747 and described by him in *Species Plantarum* in 1753. He included two species, namely *E. sessile* and *E. pedunculatum*. One of the species classified by Linnaeus under the genus *Chironia* is now included under *Exacum* as *E. trinervium*. All of the three species described by Linnaeus came from India and Sri Lanka. Other species of the genus *Exacum* were subsequently discovered by Roxburgh (1814, 1820). More of the Asian species were described in the second half of the nineteenth century, and in 1883–1884, Regel (1883) and Balfour (1884) described *E. affine* Balf. f from the island of Socotra. Some authors suggest that a more appropriate name would be *E. affine* Balf. ex Regel because the first publication on *E. affine* in 1883 was by Eduard August von Regel. The greatest diversity of species of the genus *Exacum* occurs on the island of Madagascar. Before 1955, however, only 6 species from the island were known, compared with the 39 species known at present (Riseman 2006). *E. alberti-grimaldi*, endemic from the Andrafiamena-Andavakoena region in the northern Madagascar, is the newest species described recently by Wohlhauser and Callmander (2012).

16.2.2 Ethnomedicinal Uses and Ethnobotanical Studies

Numerous ethnobotanical studies were carried out to collect information on the traditional use of medicinal plants by rural and tribal communities in the regions where plants occur of the genus *Exacum*. Information on the use of medicinal plants was collected through interviews, discussion, and field observation with herbal healers and knowledgeable elderly people. Tribal communities are often illiterate and the ethnic knowledge of the medicinal plants is traditionally passed on from one generation to another without documentation. Much of this wealth of information is lost as traditional culture gradually disappears. Hence, there is an urgent need to record and to preserve the ethnic knowledge relating to medicinal plants and the importance of scientific ethnobotanical studies. One of the ethnobotanical surveys (Karuppusamy 2007) was carried out in the Sirumalai Hills of southern India to collect the traditional knowledge of Paliyan tribes which inhabit this area. *Exacum pedunculatum* L. is one of about 90 species used as medicinal plants, whole plant bing used to treat fever with dysentery. Based on the information obtained during the ethnobotanical studies in the Melghat forest (Amravati district of Maharashtra state, India), Tambekar and Khante (2010) selected about 40 species used by the traditional herbal healers for treatment enteric infections, such as diarrhoea, dysentery, and stomach ache. *E. pedunculatum* was mentioned as a medicinal plant used not only as a febrifuge but also as a bitter tonic and anthelmintic. This species was also noted by Khare (2007) as a plant with antigout properties used in the traditional medicine.

Exacum tetragonum Roxb., like *E. pedunculatum*, is used to treat fever and stomach disorders (Sarmah et al. 2008). The information about the use of this species was obtained from the Chakma community living in the northwestern periphery of Namdapha National Park in Arunachal Pradesh (India), the whole plant being used to prepare medicinal extracts. Similarly to the Chakma community, the tribes of the Purulia district (West Bengal, India) also use *E. tetragonum* as a febrifuge (Dey and De 2012), but in this case, only plant roots are used and the medicine is administered orally as a paste.

Exacum wightianum Arn. is a plant used in traditional Indian medicine to treat inflammation (Baluprakash et al. 2011b).

The region of Kumara Parvatha near Kukke Subramanya (Mangalore, Karnataka, India) is rich in ayurvedic medicinal plants. During one of the medico-botanical surveys, 44 plant species were collected and described (Shiddamallayya et al. 2010). *Exacum bicolor* Roxb. was one of the plants and was mentioned as a tonic and stomachic. Lingaraju et al. (2013) made their ethnopharmacological survey in a different part of Karnataka, namely in Kodagu district. Their studies lasted two years (August 2010–September 2012) and revealed the ethnobotanical information of 126 plant species. *E. bicolor* was described as a plant used to treat asthma. The preparation from the whole plant was suggested to be taken with honey. In Kannur and Wayanad districts (Kerala, India), *E. bicolor* is used for the treatment of many diseases such as eye and skin problems and

stomachic and urinary disorders (Jeeshna and Paulsamy 2011b). *E. bicolor* is also mentioned as an antidiabetic herb (Sreelatha et al. 2007).

The content of the traditional medicines is sometimes variable and even doubtful. The Indian ayurvedic herb Kade-chirayet is one of such controversial medicines (Upadhye et al. 1991). Kade-chirayet is used as a tonic and febrifuge by local people from the areas of Western Ghats, from Pune, and from neighboring districts (India). Five different plant species collectively known as Kade-chirayet are used interchangeably: four plants from the family *Gentianaceae* (*E. bicolor*, *Swertia angustifolia, S. decussata*, *Enicostemma littorale*), and one plant belonging to the family *Acanthaceae* (*Andrographis paniculata*). All those species can be used to treat fever and as a tonic (all are bitter), but they do not act exactly in the same way. They also have specific activities. The example of the traditional herb, Kade-chirayet, demonstrates the importance of ethnobotanical and taxonomic studies (Upadhye et al. 1991). *E. bicolor* is also mentioned as one of the plants used to substitute or adulterate *Gentiana kurroo* (Behera and Raina 2012). This causes intentional or unintentional reduction of the drug potency.

16.2.3 Biological Activities

The large increase in the infections caused by *Candida albicans* and the growing number of resistant strains have resulted in the need to search for the new effective drugs, also of plant origin. On the basis of ethnobotanical data, 20 Indian plant species were selected and their anticandida potential investigated (Salkar et al. 2013). *E. bicolor* was one of the seven plant species showing significant activity against *Candida*. The results of the prior investigation (Paulsamy and Jeeshna 2011) also demonstrated the activity of *E. bicolor* extracts against *Salmonella paratyphi* A and *Cladosporium* sp.

Based on the ethnobotanical studies, extracts from 40 plant species used in the traditional medicine of the Amravati district (Maharashtra state, India) to treat enteric infections were tested to determine their antibacterial activity (Tambekar and Khante 2010) against pathogens such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterobacter aerogenes*, *Salmonella typhi*, *S. typhimurium*, *S. paratyphi*, *Proteus vulgaris*, *Klebsiella pneumoniae*, and *Shigella flexneri*. The extract from *E. pedunculatum* showed moderate/mild antibacterial potential. The results obtained by another research group (Mahida and Mohan 2006) also indicated the antibacterial activity of *E. pedunculatum* extracts. The effects against some of the pathogens were comparable with antibiotics.

Baluprakash et al. (2011b) tested the anti-inflammatory activity of extracts of *E. wightianum* using the carrageen an induced rat paw edema method. Their results suggested that the methanolic extract exhibited an effective anti-inflammatory activity mediated via both inhibition of the cyclooxygenase cascade and by blocking the release of histamine, serotonin, and kinins. The results also support the use of *E. wightianum* in the traditional Indian medicine to treat inflammation.

Exacum affine Balf. f. ex Regel is used as a medicinal plant in traditional medicine in Yemen and there have been some studies conducted at the University of Sana'a in Yemen to determine the pharmacological effects of *E. affine*, i.e., its antiviral, antibacterial, and anticancer activities (Mothana and Lindequist 2005; Mothana et al. 2006, 2007). It has been confirmed that the antiviral action is the principal activity of *E. affine* (Mothana et al. 2006).

Aqueous and methanolic extracts were used in the study on antiviral activity, prepared from 25 plant species of plants, including *E. affine*, which originated from the island of Socotra. The plants tested are used in Yemeni traditional medicine to treat skin and respiratory tract infections and other viral diseases. *E. affine* extracts exhibited significant activity against the influenza virus A/WSN/33 (H1N1) and herpes simplex virus type 1 (HSV-1 KOS). The herpes virus was more sensitive to the extracts than the flu virus, and 17 out of all the species studied were found to exhibit activity against the HSV-1 virus. The antiviral activity of *E. affine* can most likely be attributed to compounds such as phenolic acids and tannins (Mothana et al. 2006).

Extracts from 25 Socotra plants were prepared in order to study antibacterial properties. They were tested for their activity against Gram-positive and Gram-negative bacteria, including *Staphylococcus aureus*, *Micrococcus flavus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus subtilis*, as well as the multi-resistant *Staphylococcus* strains *S. aureus*, *S. epidermidis*, *S. haemolyticus*. However, *E. affine* did not exhibit antibacterial activity against the microorganisms evaluated (Mothana and Lindequist 2005).

In analysis of the antitumor effect of *E. affine* and other Socotra species, five human cancer cell lines were used: two of lung cancer lines, two of urinary bladder cancer lines, and one of breast cancer. Extracts of *E. affine* did not exhibit significant cytotoxic activity (Mothana et al. 2007).

16.2.4 Secondary Metabolites

Five groups of plant secondary metabolites were reported from *Exacum* species,there being iridoids, phenolic compounds (phenolic acids and acetophenone derivatives), flavonoids, and volatile constituents. Xanthones have not been reported from *Exacum* (Hegnauer 1966; Daniel and Sabnis 1978; Jensen and Schripsema 2002).

16.2.4.1 Volatile Constituents

The volatile constituents were analyzed only for *E. affine* Balf f. ex Regel by means of two different analytical methods (headspace analysis and hydrodistillation). Buchbauer et al. (1994) examined the volatile constituents of the flowers of *E. affine*

(the variety "Blithe Spirit" with white flowers) obtained by dynamic headspace sampling and in the form of the essential oil by using GC-FID, GC-FTIR-MS and a GC-sniffing technique. Freshly harvested flowers were used for the analysis. The flowers, the headspace, and the essential oil were evaluated olfactorially by perfumers before the analytical procedure. The headspace concentrate possessed a sensoric quality closer to that of the flower compared to the essential oil. Buchbauer et al. (1994) observed that the flowers and the headspace concentrate possess a characteristic lily-of-the-valley-like, fresh, attractive floral fragrance, while the essential oil shows only a weak floral odor with a distinct vegetal fragrance. The results showed significant differences in the composition of volatiles. The headspace constituents and the essential oil volatile constituents differ qualitatively and quantitatively. Forty-two compounds (excluding fatty acids and fatty esters) were identified in the essential oil. The main components were limonene (12.3 %), α-pinene (7.9 %), and camphor (9.2 %). A further eight compounds were in the amounts of 2.1–3.8 %. Seventeen compounds were in the amounts of 1–2 %; ten compounds were in amounts below 1 %, and four compounds in trace amounts. Thirty-four compounds were identified in the headspace concentrate. As for the essential oil, the major components were also the three compounds: limonene, α-pinene, and camphor, but they were present in different quantities of 18.7, 9.2, and 6.8 %, respectively. An additional twelve compounds were present of 2.1–4.7 %, seven compounds were of 1–2 %, while nine compounds were in amounts below 1 % and three compounds were in trace amounts.

16.2.4.2 Iridoids

The iridoids (mainly secoiridoids) are present universally in the family *Gentianaceae*, with a predominance of gentiopicroside and/or swertiamarin (Jensen and Schripsema 2002). Gentiopicroside has been examined in the two *Exacum* species: *E. affine* (Kuwajima et al. 1996) and *E. tetragonum* (Das et al. 1984). The compound was isolated from the fresh aerial parts of *E. affine* cultivated as an ornamental plant in Japan (Kuwajima et al. 1996). In addition to gentiopicroside, the second iridoid glucoside, 2′-*O*-*p*-coumaroylloganin, was also isolated from *E. affine* by comparison of UV, IR, ^{1}H NMR, and ^{12}C NMR spectra with published data. Das et al. (1984) isolated from *E. tetragonum* two secoiridoids: gentiopicroside and the methyl ester of methylgrandifloroside. The identities of both compounds were confirmed by ^{1}H NMR and ^{13}C NMR. Delaude (1984) found gentianine in whole plants of *E. quinquenervium*. Gentianine is an iridoid compound of an alkaloid nature. What may be formed from gentiopicroside and may be considered an artifact?

Gentiopicroside, bitter compound, has mainly gastro-stimulant activity, typical for plants of the *Gentianaceae*.

16.2.4.3 Acetophenone Derivatives

It was reported by the Minoo Park Insectarium in Osaka that lesioned parts of the *Exacum affine* Balf f. ex Regel plants showed remarkable insect attractivity to males of the giant danaid butterfly, *Idea leuconoe*. Matsumoto (1994) isolated the chemical compound which was responsible for such an action and identified the compound as paeonol (Fig. 16.1) by EIMS, UV, and ^{1}H NMR spectra. Matsumoto (1994) examined the concentration of paeonol in different parts of *E. affine*. The greatest amounts were detected in roots (0.40 and 3.3 %; fresh and dry weight, respectively), stems (0.24 and 2.6 %, respectively), and flowers (0.20 and 2.1 %, respectively), with the lowest concentration in leaves (0.08 and 0.6 %, respectively).

Kuwajima et al. (1996) isolated two acetophenone derivatives from fresh aerial parts of *E. affine*. Both compounds were paeonol (2-hydroxy-4′-methoxyacetophenone) glycosides: namely; glucopaeonol and 2-*O*-primeverosylpaeonol. The structures of the chemical compounds were identified on the basis of UV, MS, ^{1}H NMR, and ^{13}C NMR spectra.

Paeonol increases cortical cytochrome oxidase and vascular actin and improves behavior in rat model Alzheimer's disease. This chemical also reduced cerebral infarction involving superoxide anions and microglia activation in ischemia-reperfusion injured rats (Kuwajima et al. 1996).

16.2.4.4 Phenolic Acids (Figs. 16.2, 16.3)

Only a few publications relate to the phenolic acid content in species of the genus *Exacum*. Daniel and Sabnis (1978) investigated the two *Exacum* species, *E. bicolor*

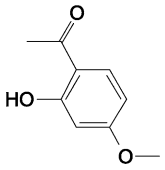
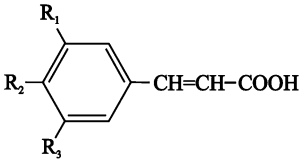


Fig. 16.1 Paeonol—the acetophenone derivative found in *Exacum affine* Balf. f. ex Regel



1: R_1=R_2=R_3=H
2: R_1=R_3=H; R_2=OH
3: R_1=H; R_2=R_3=OH
4: R_1=H; R_2=OH; R_3=OCH_3
5: R_1=R_3=OCH_3; R_2=OH

Fig. 16.2 Cinnamic acid and cinnamic acid derivatives found in the genus *Exacum*: cinnamic acid (**1**) and *p*-coumaric (**2**), caffeic (**3**), ferulic (**4**), and sinapic (**5**) acids

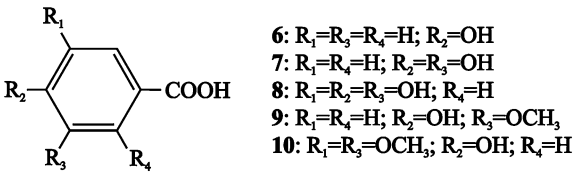


Fig. 16.3 Examples of benzoic acid derivatives found in the genus *Exacum*: *p*-hydroxybenzoic (**6**), protocatechuic (**7**), gallic (**8**), vanillic (**9**), and syringic (**10**) acids

and *E. pedunculatum*. As a result of qualitative analysis, four phenolic acids were found in *E. bicolor* (vanillic, p-hydroxybenzoic, protocatechuic, and p-coumaric acids) and six compounds in *E. pedunculatum* (vanillic, syringic, p-hydroxybenzoic, protocatechuic, p-coumaric, and ferulic acids) (Figs. 16.2, 16.3). Jeeshna and Paulsamy (2011a) estimated quantitatively the content of chlorogenic acid in *E. bicolor*. Fourteen phenolic acids were found in *E. affine* herb (protocatechuic, gallic, gentisic, chlorogenic, p-hydroxybenzoic, vanillic, caffeic, syringic, p-coumaric, ferulic, sinapic, salicylic, o-coumaric, rosmarinic; Skrzypczak-Pietraszek unpublished data). Some of them were only in the bound form and were detected after hydrolysis. The main compound was protocatechuic acid.

The antioxidant activity of phenolic acids results from their various mechanisms of action detailed by Breinholt (1999):

- agents chelating metal ions of enzymes that catalyze oxidation reactions,
- inhibitors of oxidases,
- stabilizers of free radicals produced in oxidative reactions, by hydrogenation or complexation,
- terminators interrupting radical chain reactions,
- compounds with reducing properties can donate an electron or hydrogen atom, and
- compounds that bind free radicals can stabilize or delocalize an unpaired electron.

Phenolic acids have long been used as natural medicines in the treatment of various disorders. The antioxidant activity of phenolic acids results from the chemical structure of their molecule, more precisely, from the number and arrangement of the functional groups. The number of the methoxy groups is of significance for the compounds that have only one hydroxyl group. The more methoxy groups there are in the molecule, the stronger the antioxidant activity of the compound. The highest antioxidant properties are possessed by ferulic, caffeic, and p-coumaric acids (Breinholt 1999; Khadem and Marles 2010).

Studies have shown that consumption of phenolic acids has a positive effect on the human body. For example, there is evidence of a fall in mortality caused by cardiovascular diseases, reduction in the incidence of atherosclerosis as a result of providing many natural compounds, mainly hydroxycinnamic acids, which inhibit peroxidation of cell membrane lipids, protect low-density lipoproteins (LDL) from

oxidation, and raise the level of the "good" cholesterol, HDL (high-density lipoproteins). Furthermore, phenolic compounds affect the central and peripheral nervous system. This action may result from the affinity of these compounds for GABA-benzodiazepine receptors and their stimulation. The results of recent analyses indicate a positive impact on the reduction in CNS injury during cerebral ischaemia. This is due to the modulation of the enzyme, nitric oxide(II) synthase (NOS), and antioxidant activity (Breinholt 1999).

In recent years, the antitumor activity of phenolic acids has also been investigated, since they are used in the prevention of cancer. This activity is mainly characteristic of hydroxycinnamic acid derivatives, which have the ability to inhibit the growth of tumors and prevent the formation of nitrosamines, which are mutagenic compounds. The chlorogenic, ellagic, ferulic, gallic, and caffeic acids have the ability to stop the carcinogens that are formed through metabolism of some carcinogenic substances. The ferulic and coffee acids are considered the most important inhibitors of neoplastic diseases, and the products of their degradation (8,5 dihydrobenzofurans) exhibit cytotoxic activity against leukemia cells, breast cancer, and colon cancer (Tanaka et al. 2011).

Phenolic acids also exhibit antiviral, antibacterial, and antifungal activity (Cueva et al. 2010). Their mechanism of action appears to be associated with increased cell membrane permeability due to changes in membrane potential, which results from the dissociation of phenolic acids. Antimicrobial activity is used mainly in the research on new food preservatives.

16.2.4.5 Flavonoids

Few studies have been carried out to analyze the content of flavonoids in the species of the genus *Exacum*. Linarin (acacetin-7-*O*-β-rutinoside) was found in *E. macranthum* (Gunatilaka et al. 1983). Daniel and Sabnis (1978) reported the presence of two flavonoids in *E. bicolor* (apigenin and luteolin) and two compounds in *E. pedunculatum* (luteolin and diosmetin). The content of luteolin in *E. bicolor* was estimated quantitatively (Jeeshna and Paulsamy 2011a).

Flavonoids (Fig. 16.4) belong to the large and important group of plant-derived compounds exhibiting a wide spectrum of pharmacological properties, including antioxidant, anti-inflammatory, hepatoprotective, diuretic, sedative, estrogenic, and others (López-Lázaro 2009). Flavonoids are common constituents of numerous plants used in traditional and modern medicine to treat a wide range of illness. Those compounds do not have only a therapeutic, but also a preventive potential associated with a reduced risk of developing some diseases such as cancer or cardiovascular and neurodegenerative disorders. The results of numerous studies suggest that the antioxidant activity of flavonoids plays an important role in their medicinal and protective properties. Numerous studies in vitro have revealed that

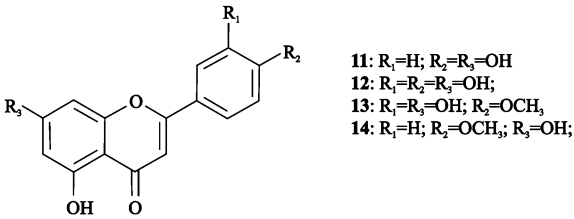


Fig. 16.4 Flavonoids (flavone derivatives) found in the genus *Exacum*: apigenin (**11**), luteolin (**12**), diosmin (**13**), and acacetin (**14**)

luteolin exhibits a wide range of biological effects, such as antioxidant, anti-inflammatory, antimicrobial, and anticancer activities (López-Lázaro 2009).

Linarin exhibits some interesting pharmacological activities, such as sedative and sleep-enhancing properties and acetylcholinesterase inhibitory activity.

16.3 Biotechnology of the Genus *Exacum* and Its Applications

16.3.1 In Vitro Propagation

16.3.1.1 *Exacum Affine* Balf. f. ex Regel (Fig. 16.5)

The species *Exacum affine* is usually propagated from seed. This method, however, is not effective, and more efficient ways of propagation of the species are being sought. Improved results involve propagation in vitro.

Micropropagation is an excellent way to obtain a large number of shoots in a limited time. The method has been used by Torres and Natarella (1984). Tissue cultures were initiated from stem fragments and grown on MS medium (Murashige and Skoog 1962). Following the addition of growth regulators (α-naphthaleneacetic acid i.e., NAA and cytokinins: kinetin and 2iP, in various concentrations), formation of brown callus and shoot-like structures was observed. Supplementing the medium with 0.0, 0.01, or 0.1 mg l^{-1}, NAA together with 0.2 or 1.0 mg l^{-1} cytokinin (Kinetin or 2iP) resulted in the largest number of shoots (Torres and Natarella 1984). In a study on the effects of phenylurea (CPPU) and 6-benzylaminopurine (BAP) on culture of *E. affine*, it was shown that the addition of BAP or CPPU to the growth medium increased the number and weight of shoots. This effect was especially evident at BAP concentrations of 1, 5, and 10 μM (Kapchina-Toteva et al. 2005). Growth of lateral shoots was also observed following the addition of 1, 5, or 10 μM CPPU, but to a lesser extent than after the addition of BAP.

Propagation by somatic embryogenesis is an effective method of clonal propagation. The prerequisite for this pathway of regeneration is stimulation of the growth of callus tissue and selection of embryogenic tissue. Normally, callus

Fig. 16.5 *Exacum affine* Balf. f. ex Regel. Bar = 5 mm

cultures are initiated with young, rapidly dividing explants (such as cotyledons and young leaves) and immature inflorescences and pedicels. The young parts of plants are the least contaminated microbiologically. The species *E. affine* is also known for endogenous bacterial infections, and which is why buds and pedicels were used as explants in the study by Ornstrup (1993). Explants were taken from seven varieties of *E. affine*. Mutations were not detected in those plants. Explants were cultured on MS medium semi-solidified with 0.3 % Gerlite™ with the additions of 3 % (w/v) sucrose and various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and BAP as growth regulators. It was shown that

- at a concentration of 9.0 μM 2,4-D embryogenic callus was not formed, and above 1.0 μM BAP, its development was inhibited completely.
- the most suitable combination of growth regulators for the formation of embryogenic callus was 9.0 μM 2,4-D + 0.089 μM BAP.
- formation of somatic embryos was observed after transferring the callus onto a medium without the addition of 2,4-D, or at a concentration of less than 9.0 μM 2,4-D.

Experiment also involved growing a suspension culture of embryogenic callus. The culture was obtained by washing through sieves (of different mesh sizes), the callus from semi-solid cultures, with liquid MS medium supplemented with growth regulators.

Development of somatic embryos was observed only on a medium lacking 2,4-D, or with the addition of less than 9.0 μM 2,4-D (Ornstrup et al. 1993).

Cultures of *E. affine* have also been performed at the Department of Pharmaceutical Botany of the Jagiellonian University Collegium Medicum. The cultures were initiated from seeds obtained from the Botanical Garden in Aachen and from pot-grown plants. Shoot cultures of this species were obtained on an agar-solidified MS medium

supplemented with the growth regulators: 1 mg l^{-1} BAP, 0.5 mg l^{-1} NAA, and 0.25 mg l^{-1} GA_3 (gibberellic acid Skrzypczak-Pietraszek (unpublished data).

16.3.1.2 *Exacum Bicolor* Roxb (Fig. 16.6)

E. bicolor is a popular herb used in traditional medicine to treat a range of diseases. This species is included in the endangered category because of over-exploitation. Plantlets regenerated in vitro could be used in therapeutics. Jeeshna and Paulsamy (2011a) developed a method for micropropagation of *E. bicolor*. Nodes from young and healthy branches of the plants were used as explants. The callus formation was the most effective on MS medium with BAP and 2,4-D at 1.5 and 0.9 mg l^{-1}, respectively. Callus differentiated shoots on MS medium containing BAP and NAA at 1.0 and 0.2 mg l^{-1}, respectively. Multiple shoots were obtained by subculturing the secondary explants on MS medium with BAP and GA_3 at 1.5 and 0.5 mg l^{-1}, respectively. The regenerated shoots were rooted on MS medium with IBA and NAA at 1.0 and 0.5 mg l^{-1}, respectively.

16.3.1.3 *Exacum Travancoricum* Bedd

E. travancoricum is a critically endangered plant. It is a branched woody perennial species, endemic to the south Western Ghats of Tamil Nadu, India. Its natural

Fig. 16.6 *Exacum bicolor* Roxb. (author: L. Shyamal, 2006; location: Talakaveri, Coorg, India; license: CC-BY-2.5). Bar = 1 cm

distribution is estimated at no more than 250 plants in the area of the Thirunelveli Hills. The Botanical Survey of India has recommended that special attention be paid to the propagation and conservation of this species. Moreover, because of its ornamental flowers, *E. travancoricum* has the potential to be of horticultural importance. Consequently, some studies have been undertaken to develop efficient in vitro propagation. Kannan et al. (2007) focused on the micropropagation of *E. travancoricum* using internode segments excised from young shoots. The plants used as a source of explants originated from the herbal garden of the Entomology Research Institute of Loyola College, Chennai, India, having been collected earlier from their natural habitat and established in that garden. The internode explants (0.5–1.0 cm long) were cultured on MS medium supplemented with different concentrations (0.0–3.0 mg l^{-1}) of thidiazuron (TDZ) or BAP. Rooting of regenerated shoots was attempted by transferring the shoots to MS medium with 0.0–4.0 mg l^{-1} IBA or 0.0–2.0 mg l^{-1} IAA. Direct morphogenesis of shoots was observed on the cut ends of the internode explants cultured on MS medium with TDZ or BAP with. TDZ being superior to BAP in the induction and proliferation of shoots. The medium containing 2 mg l^{-1} TDZ yielded more of shoots per explants, an average of 2.8 ± 0.2 and 86 % of the explants used produced shoots. The medium supplemented with 2.0 mg l^{-1} BAP induced only an average of 1.2 ± 0.2 shoots per explant. TDZ concentrations above 3.0 mg l^{-1} affected the formation of the basal green callus and the explants did not regenerate. Greater concentration of BAP (2.5 and 3.0 mg l^{-1}) increased the number of shoots (3.5 and 2.9, respectively), but only less than 50 % of the explants produced shoots. Kannan et al. (2007) emphasized the efficacy of TDZ may be attributed to its ability to induce cytokinin accumulation, or to enhance the accumulation and translocation of auxin within tissues. The regenerated shoots were rooted on MS medium with IAA or IBA, the optimum medium contained 3.0 mg l^{-1} IBA.

A total of eighty percentage of in vitro-obtained plantlets survived acclimatization to ex vitro conditions. Another group (Janarthanam and Sumathi 2010) described their micropropagation protocol from shoot tip explants of *E. travancoricum* to large-scale propagation. The shoot tip explants were inoculated on to MS medium with different concentrations and combinations of BAP (1.11, 2.22, 4.44, 6.66, and 8.88 μm) and NAA (0.54, 1.34, 2.69, and 5.36 μm) for shoot initiation. The proliferated shoots were transferred to ½ MS medium supplemented with IBA (0.49, 0.98, 2.46, 4.92, and 12.3 μm) for root development. All combinations of BAP and NAA influenced the formation of additional shoots on the explants. MS medium containing 4.44 μm BAP and 1.34 μm NAA was the most efficient for multiple shoot development. About 80 % of the cultured explants formed additional shoots and produced 29.3 ± 0.3 shoots per explant.

16.3.1.4 *Exacum Wightianum* Arn.

E. wightianum is an endemic medicinal subshrub. Baluprakash et al. (2011a) developed a method for its micropropagation with leaf, nodal and axillary bud

explants *E. wightianum* being used. They were cultured on MS medium supplemented with various concentrations and combinations of plant growth regulators (BAP, BAP with NAA, BAP with 2,4-D, and BAP with KIN) for callus induction. Callus on MS medium with BAP and NAA possessed the more regenerative potential than on other medium combinations. Stock callus was subcultured to obtain multiple shoots. Most shoots were obtained on MS medium supplemented with BAP (2.5 mg l^{-1}), or with BAP and NAA (2.0 and 0.5 mg l^{-1}, respectively).

16.3.1.5 *Exacum* "Styer Group"

In 2005, the name "Styer Group" was proposed for interspecific hybrids of *Exacum* originating from Sri Lanka (Riseman et al. 2005). Micropropagation of the hybrids has been carried out on MS medium supplemented with different growth regulators. Most roots were obtained when the medium contained NAA, and the lowest when it was supplemented with IBA. Additions of 2iP and BAP resulted in shoot formation, but kinetin inhibited shoot growth and bud formation. Callus was formed on medium containing BAP. There have been studies on organogenesis in this group, the aim being direct organogenesis without a callus stage. Explants were cultured on MS medium supplemented with BAP (0, 0.44, 2.22, 4.44, or 8.88 μM) and NAA (0, 0.05, 0.54, or 2.69 μM). Process of organogenesis did not occur on medium without growth regulators. When the medium contained 2.69 μM NAA and 0.44 μM BAP, root growth was clearly evident, accompanied by the presence of only limited number of shoots. A large number of shoots was obtained with 2.69 μM NAA and 8.88 μM BAP, while callus and shoots were formed with the condition of 0.05 μM NAA and 2.22 μM BAP. Supplementing the medium with 2.69 μM NAA and 4.44 μM BAP resulted in the formation of callus, roots, and shoots (Unda et al. 2007).

16.3.2 Secondary Metabolites from Shoot Cultures

E. bicolor

Jeeshna and Paulsamy (2011a) determined the content of luteolin and chlorogenicacid in regenerated plants compared with the concentration of those compounds in intact plants. Plantlets contained lower amounts of luteolin and more of chlorogenic acid than the intact plants.

E. affine

Shoot cultures (Fig. 16.7) contained the same 14 phenolic compounds as pot-grown plants, but all compounds were both in free and bound form (Skrzypczak-Pietraszek, unpublished data).

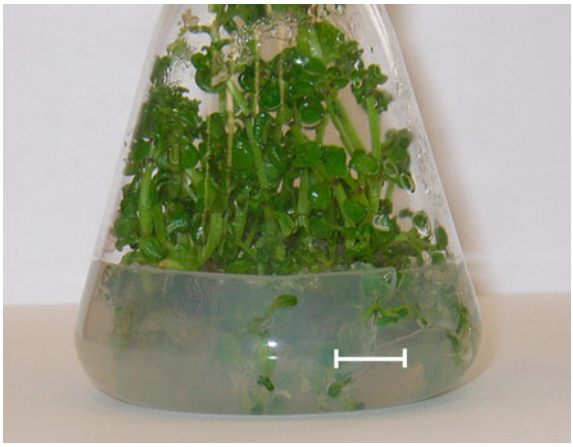


Fig. 16.7 Shoot cultures of *Exacum affine* (MS medium supplemented with BAP—1.0 mg l^{-1}; NAA—0.5 mg l^{-1}; GA_3—0.25 mg l^{-1}). Bar = 1 cm

16.3.2.1 Attempts to Improve the Accumulation of Secondary Metabolites

Phenolic acids are an important group of plant secondary metabolites with different, valuable therapeutic properties. Besides plants growing naturally, tissue cultures can be an alternative source of secondary metabolites. Their accumulation in cultures can be increased by different methods, including supplementation of culture medium with precursors, elicitors, and changing standard amounts of the medium components (Karuppusamy 2009). Skrzypczak-Pietraszek et al. (2014) investigated the influence of precursor (L-phenylalanine), elicitor methyl jasmonate (MeJA), and increased sucrose concentration on phenolic acid accumulation in agitated shoot cultures of *E. affine* (Fig. 16.8).

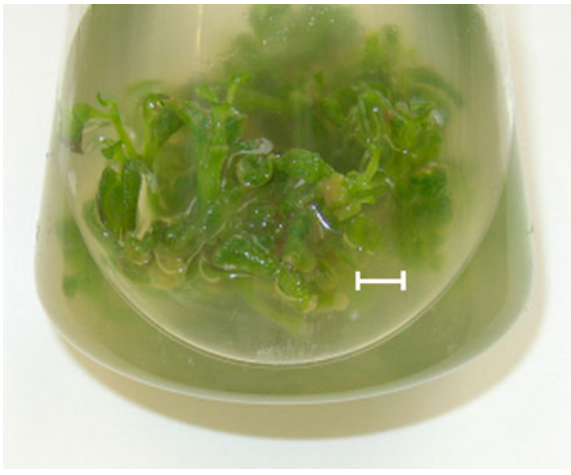


Fig. 16.8 Agitated shoot cultures of *Exacum affine* (MS medium supplemented with BAP—1.0 mg l^{-1}; NAA—0.5 mg l^{-1}; GA_3—0.25 mg l^{-1}). Bar = 1 cm

Phenylalanine (Phe) is an amino acid, the precursor of the phenylpropanoide pathway leading to the formation of phenolic acids, flavonoids, and other phenolic compounds. Phenylalanine has been used to increase the metabolite production in vitro in several different plant cultures (Arora 2011).

Methyl jasmonate, an abiotic elicitor, activates phenylalanine ammonia lyase (the enzyme that catalyzes the first step in the shikimic acid pathway, the deamination of Phe) and thus induces secondary metabolite production (Namdeo 2007).

Sucrose is one of the standard components of culture media and an important carbon and energy source for plant cells. In addition, the sucrose influences the production of secondary metabolites of the phenylpropanoid pathway (Arora 2011).

Cultures were maintained in Erlenmeyer flasks with MS medium supplemented with BAP (1 mg l^{-1}), NAA (0.5 mg l^{-1}) and GA_3 (0.25 mg l^{-1}). Variant A' contained 3 % (w/v) of sucrose (standard amount) and the other six variants (A–F) 6 % (w/v) of sucrose. After two weeks, L-phenylalanine (1.6 g l^{-1}) and/or MeJA (100 μM or 800 μM) were added to B–F variants. Variants A′ and A were treated as references. Plant materials were collected after 1, 3, and 7 days after the addition of the precursor and/or the elicitor, as were control samples. Phenolic acids were assayed in the collected biomass before and after acid hydrolysis (2 M HC1). Qualitative and quantitative analyses of phenolic acids in methanolic extracts from biomass were conducted by an HPLC method. Fourteen phenolic acids (protocatechuic, gallic, gentisic, chlorogenic, p-hydroxybenzoic, vanillic, caffeic, syringic, p-coumaric, ferulic, sinapic, salicylic, o-coumaric, rosmarinic) and cinnamic acid were found in all samples. The total content of free phenolic acids increased from approximately 0.242 to 0.635 % (2.6-fold), and the total content of phenolic acids (free and bound) from 0.712 to 1.160 % (1.6-fold). The studies show that the best variant contained 6 % (w/v) of sucrose (double amount of the standard), L-phenylalanine (1.6 g l^{-1}), and MeJA (100 μM).

Analysis of the results in the experiments showed that it is possible to increase the accumulation of phenolic acids in shoot cultures *E. affine* by adding the precursor L-phenylalanine, the elicitor (MeJA) and increasing the sucrose concentration (Skrzypczak-Pietraszek et al. 2014).

16.3.2.2 Biotransformation

Plant cell and tissue cultures are capable of performing various specific biotransformation reactions on exogenously supplied compounds. A whole range of reactions have been observed including esterification, oxidation, reduction, hydroxylation and glucosylation. The formation of glucosyl conjugates is of special interest because many groups of secondary metabolites are accumulated as glucosides in plant cells (Tabata et al. 1988; Stöckigt et al. 1995). Arbutin is the O-β-D-monoglucoside of hydroquinone. From a pharmacological point of view, arbutin has attracted much interest for two main therapeutical applications. The compound shows urethral disinfectant activity and is known as an efficient inhibitor of melanin biosynthesis in human skin. Agitated shoot cultures of *E. affine* are able to perform

the biotransformation of exogenously supplied hydroquinone to arbutin with a maximal efficiency of 65.5 % (Skrzypczak-Pietraszek et al. 2005). Such cultures are promising subjects for further investigations on optimization of the process.

16.4 Conclusions

The genus *Exacum* is poorly studied in terms of its chemical compounds. Major uses in traditional medicine, confirmed by ethnobotanical studies and some investigations on biological activities, suggest considerable pharmacological potential of *Exacum* species. Plants derived in vitro could be the source of medicines and material for isolation of pharmaceutically important compounds. The accumulation of secondary metabolites in plant tissue cultures can be improved using biotechnological methods. The volatile oil of *E. affine* has been isolated and examined with results suggesting its potential perfumery applications. Numerous *Exacum* species are endemic and often endangered, caused by overexploitation, and micropropagation can be useful in protection of those species and for introducing such plants to commercial floriculture.

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