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CHARACTERISATION OF THE ANTIMICROBIAL AND ANTIOXIDANT PROFILE OF *Phalaenopsis* ORCHID WASTES

Laura-Simona IRIMESCU[1], Celia GUERRERO OLIVARES[2], Camelia Iustina PREDA[1], Camelia Filofteia DIGUȚĂ[1], Gabriela LUȚĂ[1], Daniela BĂLAN[1], Florentina MATEI[1]

[1]University of Agronomic Sciences and Veterinary Medicine of Bucharest, Faculty of Biotechnology, 59 Mărăști Blvd, District 1, Bucharest, Romania
[2]Universitat Politècnica de València, Camino de Vera s/n, 46022 Valencia, Spain

Corresponding author email: laura.irimescu@gmail.com

Abstract

Orchids are widely known for their economic importance, but less so from a medicinal point of view. This paper aims to provide information on the antimicrobial and antioxidant potential of methanolic and ethanolic extracts obtained from orchid wastes of Phalaenopsis species, respectively dried leaves, stems and roots. Due to their high diversity, bioactive plant compounds are of particular interest. The antioxidant potential was studied by measuring the phenolics, the chlorophyll and carotenoids content; in addition, DPPH assay was used. Antimicrobial tests were done by spot method on different microorganisms (Staphylococcaceae, Bacillaceae, Enterobacteriaceae family and Candida sp.). The results show a medium to high antioxidant potentila of Phalaenopsis wastes extracts (leaves and roots) and a promising inhibitory activity of leaves extracts against Staphylococcus aureus methicillin resistant and Pseudomonas aeruginosa. Stem extracts had inhibitory activity of Bacillus cereus. This may open future possibilities of using Phalaenopsis wastes in pharmaco-cosmetic industry.

Key words: *orchids, waste, antimicrobial, antioxidant.*

INTRODUCTION

Orchidaceae family, known as orchids as the common name, is one of the largest families of flowering plants, the second family, in fact, with 28,484 species (KewWCSP, 2017). Orchids are more often met in tropics regions. The greatest number of species was encountered in Colombia. The *Phalaenopsis* spp. is the most commonly used orchid commercially and economically. Most of its parts are often left unused, which can lead to waste and, consequently, to additional ecological problems, even if it could have a potential additional value. More than 45% from flowers produced worldwide become waste (ICT integral, 2020). *Phalaenopsis* are widely used for their distinctive beauty, but many other orchids are also found as extracts in the cosmetics industry, because they have an anti-aging effect, and moisturizes the skin. Orchid's properties are also used in the Japanese female cosmetics industry for face creams that reduce the skin depigmentation (Tadokoro et al., 2010). From ethnopharmacological point of view, studies show that *Orchidaceae* family was used for a long time in different diseases like: digestive system diseases, infections, blood circulation, pain reliever (Ashraf et al., 2013). There are some antibiotics to treat skin issues (like acne for example), but it was noticed a growing antibiotic resistance from bacteria, so several plants have inhibitory activity against this kind of microorganisms, such as *Symplocos racemose, Chamomilla matricatia*, *Syzygium aromaticum, Melaleuca alternifolia, Rosmarinus officinalis* etc. Previous studies showed promising results regarding *Phalaenopsis* extracts action against bacteria (*Staphylococcus* spp*., Salmonella, E. coli*) or fungi (*Candida* spp.), and the need for more experimental studies, using different methods or method variables (Irimescu et al., 2020).

Different studies have been made in order to discover a new range form of antioxidants. They have many health benefits, preventing or even eliminating free radicals, which leads to the reduction of degenerative diseases by slowing down the aging process of cells.

Phenolic compounds, which are secondary metabolites, have applicability in the production of antioxidants. Thus, the identification of new antioxidants has become a goal. This variety of *Phalaenopsis* orchid has become in recent years, a popular potted plant worldwide, from Japan, to America and to Europe. This plant loses its vivacity after the first flowering, later turning into waste. The disposal of *Phalaenopsis* orchids has a negative impact on the environment (Minh et al., 2016). Therefore, through this study, it was sought to evaluate the antioxidant properties of orchid plant waste. Phenolic compounds exist both in free forms and can be extracted in various polar solvents, as well as in bound forms in plant cells. Therefore, various extraction techniques have been applied to examine the antioxidant capacity, total polyphenolic compounds and total flavonoids in orchids.

This study tried to emphasize that this plant has not only ornamental values, but also has important pharmaceutical values, as antioxidant properties and inhibitory effect on different bacteria or fungi species (Bhattacharjee et al., 2015). The antioxidant potential was studied by measuring the polyphenolic compounds, the chlorophyll and carotenoids content; in addition, DPPH assay was used. Antimicrobial tests were done by spot method on different microorganisms (*Staphylococcaceae, Bacillaceae, Enterobacteriaceae* family and *Candida* sp.).

MATERIALS AND METHODS

Collection of plant material

Tria's Flower Shop, located in Greenhouse Băneasa, Romania donated the *Phalaenopsis* orchid waste, in order to be exploited to the full potential. The entire unused plants were keptincontrolled storage conditions, before they were given away by Tria's Flower Shop.

Extracts' preparation

The *Phalaenopsis* orchid species were separated into groups of leaves, stems and roots. After cutting, the tissue was dried. In a separate room, at a constant temperature of 37°C, the drying process took place for 7 days. The small cut pieces of orchid were grounded. Any impurities that could have contaminated the final sample, including bark or any other materials that may interfere with the results, were washed off with purified water, before the drying process of the orchid parts. In order to obtain the crude extract, the plant material was powdered (leaves-L, roots-R and stems-S) and mixed in a stopper bottle (Erlenmeyer) with 70% Ethanol (1:10 ratio). Each group of cut orchid was mixed with Ethanol (Nicolcioiu et al., 2017) and placed in the plate mixer for 60 minutes, at a controlled temperature of 30°C, at 150 rpm. After obtaining the crude extracts, these were filtered through a filter paper. The maceration was the method of achieving the crude extraction. The same method was used to obtain the methanol extracts from parts of *Phalaenopsis* orchids (Jagannath et al., 2016).

Antioxidant compounds

The total polyphenols were determined by an adapted Singleton method (Singleton et al., 1999; Catana et al., 2020) which is a spectrophotometric method that is based on the reduction of phosphowolframates and phosphomolybdates in the Folin-Ciocâlteu reagent. The results were presented in this paper, in milligrams equivalent gallic acid (GAE) present in one gram of fresh substance (S.P.). To calculate the results, according to the working method, a standard curve was developed.

The spectrophotometric method has permitted to determine the content of **chlorophyll pigments** (Lichtenthaler, 1987). The chlorophyll pigments were extracted with 80% acetone, as solvent. The extinction values were transformed into concentration values based on the following formulas:

Chlorophyll a concentration = (12.25 x E663 - 2.79 x E646)
Chlorophyll b concentration = (21.50 x E646 - 5.10 x E663).

The final result was expressed in mg/g.

The **carotenoid pigments** measurement was also done by a spectrophotometric method (Tănase, 1991). The appearance of the peaks characteristic of the presence in the medium of β-carotene at wavelengths between λ = 400-500 nm is observed. Qualitative analysis is based on the position of the peaks (Neamţu, 1996). For one hour, it was performed the extraction of carotenoids in the dark, under stirring.

The samples extinctions were determined at 451 nm (Britton et al., 1995). The extinction values were transformed into concentration values by means of a standard curve made for standard β-carotene solutions having concentrations between 20-100 (μg/ml). The final result was expressed in mg/g sample.

Total antioxidant capacity by diphenyl-picrylhydrazyl radical spectrophotometric method (DPPH)

The method using DPPH as a stable free radical was developed by Blois (Blois, 1958) and developed by Brand-Williams et al. (Brand-Williams et al., 1995; Bondet et al., 1997) to be used in studies using complex matrices such as biological material. DPPH is a stable free radical which has an intense, purple colour with a maximum absorption around 515 nm. Brand-Williams and co-workers, introduced the notion of "effective concentration" (EC_{50}) for the interpretation of the results obtained by the DPPH method. For this purpose, extracts of different concentrations are analysed in terms of their ability to transform the DPPH radical. For each case the percentage of free radical transformation is calculated according to the formula:

$$\% \, RSA = (1 - [D.O._{sample} / D.O._{control\ (pt.\ T = o)}])/100$$

The dependence of the transformation percentage (RSA%) on the extract concentration is plotted and the EC_{50} parameter is calculated.

In our study we used the term IC_{50} because we measure the inhibition level. EC_{50} defines in general the concentration of a drug and "Inhibitory Concentration" (IC) is more appropriate in this case (both are defining the same method).

All experiments were performed in triplicate.

Antimicrobial activity

For the inhibitory effect of different *Phalaenopsis* plant parts extracts on bacteria and fungi strains the spot-diffusion method was used.

Test microorganisms used

To test the antimicrobial activity of *Phalaenopsis* extracts, more potentially pathogenic strains of *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Candida, Salmonella, Bacillus cereus* and *Pseudomonas* (Table 1) were selected. These pathogenic microorganisms were provided by the USAMV of Bucharest, Faculty of Biotechnologies.

Culture media

The culture medium used for *Staphylococcus* and *Enterobacteriaceae* was the TSA (Tryptic Soy Agar) medium. For the *Candida* strains, culture media was PDA (Potato Dextrose Agar). For the *Salmonella* strain, culture media was XLD (Xylose Lysine Deoxycholate). For 15 minutes, all the media were introduced into the autoclave at 121°C.

Table 1. Microorganisms used to test the antimicrobial activity of *Phalaenopsis* spp. wastes extracts

No.	Microorganisms
Bacteria	
1.	*Pseudomonas aeruginosa* ATCC 15442
2.	*Bacillus cereus*
3.	*Salmonella typhi* ATCC 14028
4.	*Escherichia coli* ATCC 8739
5.	*Staphylococcus aureus* ATCC 43300 MRSA
6.	*Staphylococcus aureus* ATCC 33592
7.	*Staphylococcus aureus* ATCC 6538 MSSA
8.	*Staphylococcus epidermidis* ATCC 51625 MRSE
9.	*Staphylococcus epidermidis* ATCC 12228MSSE
Fungi	
10.	*Candida albicans* ATCC 10231
11.	*Candida glabrata* ATCC 2001
12.	*Candida parapsilosis* ATCC20019
13.	*Candida tropicalis* ATCC 44508
14.	*Candida krusei* 2016 MI 41
15.	*Candida guilliermondii* MI 40

The **drop diffusion** (spot method) test method was used to measure the areas of inhibition, with the determination of antimicrobial activity (Purcaru et al., 2015). For the drop diffusion test, the inoculum was prepared in fresh culture in specific liquid media. The fresh culture strains were prepared by inoculation on liquid media. According to the working method, the spreading technique is used to inoculate the pathogenic strains on the surface of the culture media distributed in Petri dishes. The inhibition is shown by halo-formation and its size should be measured.

RESULTS AND DISCUSSIONS

Antioxidant compounds

Polyphenols are usually defined as antioxidants, and protect, together with different vitamins, against tissue oxidation. The polyphenolic content and concentration in *Phalaenopsis* waste proves that it can be used for dietary, skin care, and maybe health purposes. The highest concentration of polyphenols was found in 70% ethanolic extract from leaves as it can be seen in Figure1 and can be studied further more for health and food industry implementation. Other studies (Minh et al., 2016) shown that orchid extracts contain smaller quantities of polyphenolic compounds, but the extracts were dissolved only in methanol, while in this study it was also used ethanol as an extracting solvent, with better results. According to Minh et al. (2016), the highest polyphenolic concentration was found in roots 4.01 mg GAE/g DW (DW = dry weight), in current study, the highest polyphenolics content was found in leaf extracts and is 6.72 mg GAE/g DW. Same author, made another study in 2017 (Minh et al., 2017) using *Phalaenopsis* Sogo Yukidian "V3" hybrid, and maximum phenolic values were found in stems: 5.092 mg GAE/g DW.

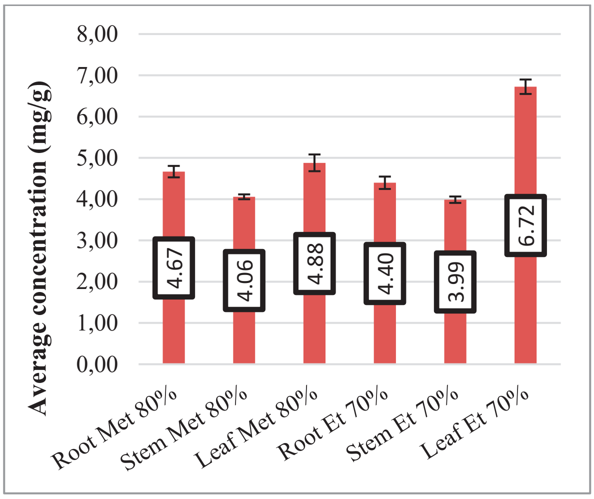


Figure 1. Average polyphenol concentrations in different *Phalaenopsis* plant parts extracts

Carotenoids play an important role as antioxidant in the human body. Carotenes can enhance immune system, because of the provitamin A that can be converted into vitamin A (Anthony, 2018). It can be seen that the leaves have the highest concentration of carotene, for this time, both methanol 80% and ethanol 70% extracts as it can be seen in Figure 2. Regarding other studies about carotenoid content in *Phalaenopsis* extracts, the information is pretty scarce, only Nguyen et al. (2018), offered values of 0.79 mg/g DW in the leaf extracts, significantly higher than 0.038 mg/g DW found in current study. Difference can be made by the fact that in Nguyen et al. (2018) study, the *Phalaenopsis* flowers received constant treatment of a fertilizer solution (N: P_2O_5: K_2O, 20: 20: 20).

The orchid plants in our study were already considered waste and did not receive any fertilizer or special care for more than 30 days before use.

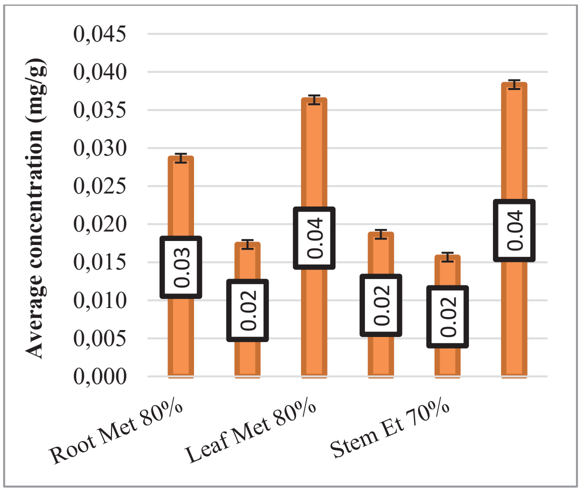


Figure 2. Average carotene concentrations in different *Phalaenopsis* plant parts extracts

Chlorophyll is the main pigment that is used by plants to transform light into energy (photosynthesis) (Soni et al., 2018). Antioxidant capacity is 1000 times superior to xanthins: caffeine, theophylline, theobromine, (tea, coffee, maté, chocolate) (Land Art, 2021).

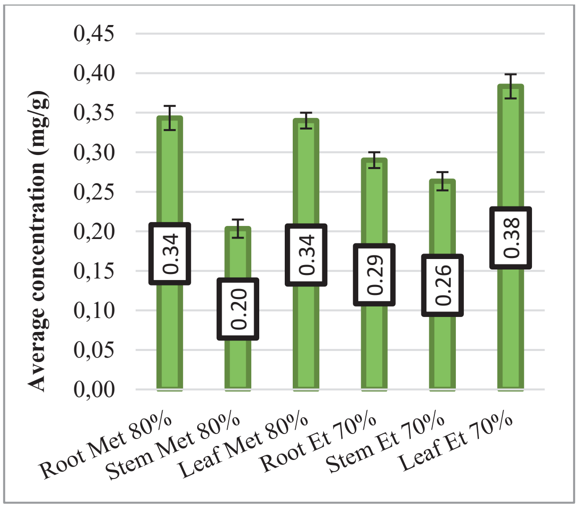


Figure 3. Average chlorophyll concentrations in different *Phalaenopsis* plant parts extracts

As it can be predicted, leaves extracts have the highest concentration of chlorophyll, but, a curious value that will need investigation is that methanol root extracts have a high concentration, as it can be seen in Figure 3.

The chlorophyll concentration found in *Phalaenopsis* was also measured by Nguyen et al. (2018), and reaching a maximum of 3.15 mg/g DW in the leaf extract of the hybrid called "City More", this value being considerably higher than the highest of the current study: 0.38 mg/g DW, found of course in leaves. In this study the methanolic extracts had the maximum chlorophyll value in leaves, meaning 0.34 mg/g concentration.

IC_{50} inhibitory concentrations

In order to calculate the IC_{50} value, the concentration of the sample needed to inhibit 50% of radical had to be calculated (Rashmi et al., 2015). The antioxidant activity of samples gets bigger as the IC_{50} value is smaller (Li et al., 2009). It can be noticed that the highest antioxidant activity is proved to be found for the ethanolic leaves extract as shown in Figure 4.

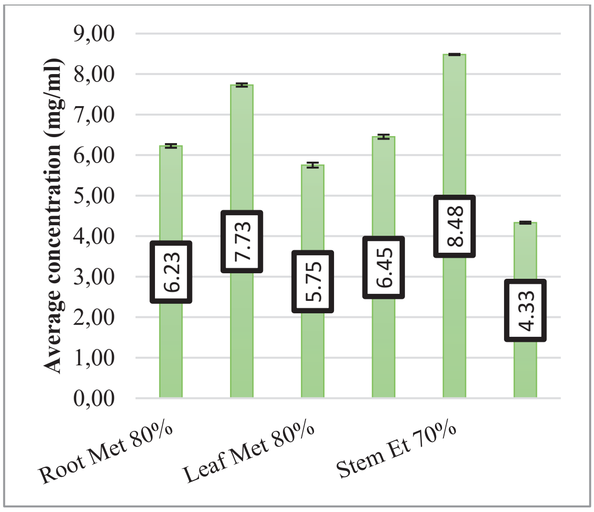


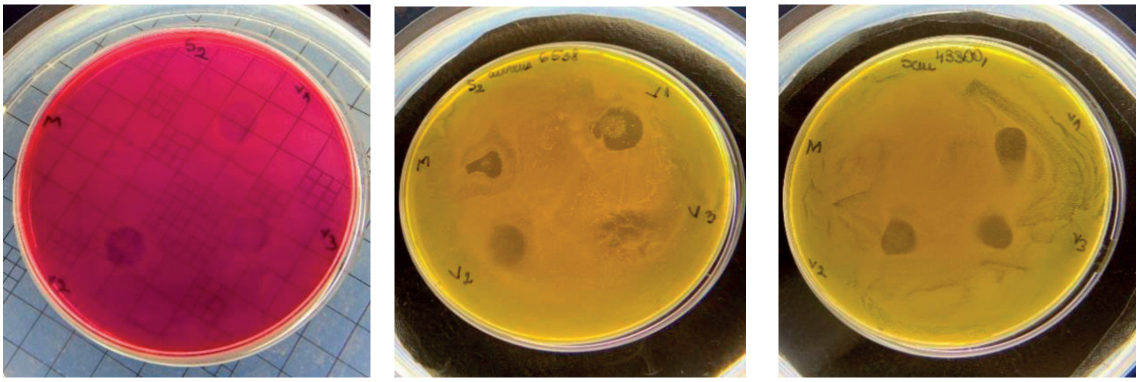
Figure 4. IC50 inhibitory concentrations of DPPH radical-scavenging activities for different *Phalaenopsis* plant parts extracts

In his study Nguyen et al. (2018), found a minimum IC_{50} concentration of 8.01 mg/ml in the methanolic leaves extracts of a *Phalaenopsis* hybrid "City More". Current work concluded that the minimum IC_{50} value was 4.33 mg/ml in leaves used as ethanolic plant extract. Methanol plant extracts in this study have a minimum of 5.75 mg/ml in leaf extract. Minh et al. (2017), studied also the IC_{50} levels for waste *Phalaenopsis* orchids and found the lowest value in leaves ethyl acetate extracts: 0.070 mg/ml, but also in stems chlorophorm extracts (0.175 mg/ml).

In addition, other tests should be performed to prove any antioxidant potential of such extracts for a higher value of new products (Paul et al., 2013). Most of the studies regarding antioxidant activity of *Phalaenopsis* are made not on waste, but on fresh plants. This fact cannot permit fertilizers to completely exit the plant's metabolism and, in this way, can influence experimental values.

Antimicrobial activity

A clear inhibition halo (inhibition examples can be seen in Figure 5.) which denotes a medium inhibitory activity, could be observed for ethanolic leaves extract on strains of *Candida*: *C. tropicalis* and *C. guilliermondii*. In the case of ethanolic root extract, high inhibitory activity has been noticed against *C. krusei.* Low inhibitory activity was registered in the case of *C. albicans* and *C. parapsilopsis* for all extracts. Bacterial activity was inhibited in different degrees especially by the leaves extracts. It was noticeable that the methicillin resistant *Staphylococcus aureus* was significantly inhibited by the leaves ethanolic extract (Table 2)., as well as the methicillin sensible strain. *Pseudomonas aeruginosa* strain was sensible to the ethanolic leaves extract in a high degree, while it was resistant to the compounds of the roots extracts. As exception, stem extract inhibited the growth of *Bacillus cereus*; further investigation on potential inhibitory compounds are requested.



a. b. c.

Figure 5. Examples of halo inhibition for: a - *Salmonella*; b - *Staphylococcus aureus* 6538; c - *Staphylococcus aureus* 43300

Table 2. Inhibitory effect of *Phalaenopsis* extracts using spot diffusion method

Domain	Microorganism	Inhibition halo ethanolic extracts			Inhibition halo methanolic extracts		
		Leaves (V1)	Roots (V2)	Stems (V3)	Leaves (V1)	Roots (V2)	Stems (V3)
Bacteria	*P. aeruginosa* ATCC 15442	+++	+	-	+++	-	-
	B. cereus	+	++	+++	+	+	+++
	S. typhi ATCC 14028	+++	++	+	+++	++	+
	E. coli ATCC 8739	++	+	-	+++	-	++
	S.aureus ATCC 43300 MRSA	+++	++	++	+++	++	++
	S. aureus ATCC 33592	+++	+	-	+++	+	-
	S. aureus ATCC 6538 MSSA	+++	++	+	+++	+	+
	S. epidermidis ATCC 51625 MRSE	++	+	-	++	+	-
	S. epidermidis ATCC 12228 MSSE	++	+	+	++	+	+
Fungi	*C. albicans* ATCC 10231	-	+	-	-	+	-
	C. glabrata ATCC 2001	+++	++	-	+++	++	-
	C. parapsilosis ATCC20019	-	-	-	-	-	-
	C. tropicalis ATCC 44508	+++	++	-	+++	++	-
	C. krusei 2016 MI 41	++	+++	+	+++	++	+
	C. guilliermondii MI 40	+	+	+	+	+	+

Legend: - = no inhibitory activity; + = low activity: 0.1-0.9 cm (indicates possible reactivity, halo size almost unnoticed); ++ = average activity: 1.0 - 1.9 cm (visible reactivity, small halo size); +++ = increased activity: > 2 cm (large halo size, indicates increased reactivity)

This preliminary study has revealed that ethanolic extracts made of leaves of *Phalaenopsis* wastes have potential antimicrobial activity. The antimicrobial activity of stems extract against all tested microorganisms was very low, while in the case of leaves and roots extracts, further antimicrobial activity should be performed.

In another study, Rashmi et al. (2015), found promising results of orchid extracts against *Bacillus subtilis*, *Escherichia coli* and *Salmonella typhi*. Similar researches, testing separate parts of *Phalaenopsis* extract effects, were not encountered so far, only other species of orchids were used for testing antimicrobial activity.

CONCLUSIONS

Polyphenols were found in highest concentrations in leaves, as expected. It was also noticed that leaves have an elevated concentration of carotenoid contents, as well as in chlorophyll, close to the content of the fresh cut orchids reported by other. Regarding IC_{50} concentrations obtained through DPPH assay, the leaves have the highest antioxidant activity, while the stem extracts show almost half antioxidant activity in relation to the leaves extracts.

This preliminary study has revealed that ethanolic extracts made of leaves of *Phalaenopsis* wastes have potential antimicrobial activity, with an inhibitory effect on pathogens like methicillin resistant *Staphylococcus aureus, E. coli, Pseudomonas,* and different *Candida* species. The antimicrobial activity of stems extract against most tested microorganisms was very low (except *Bacillus cereus*), while in the case of leaves and roots extracts, the activity was significantly higher.

Final conclusion is that leaves are the most valuable part of *Phalaenopsis* plant, and they can be used for their great inhibitory effects on bacteria and fungi. Antioxidant activity confirms that the Moth Orchid (especially the ones considered waste) can be used in cosmetic industry. Due to previous reasons, it can be stated that orchid waste, once a recycling problem, can represent in the future an important ecological and economical gain.

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