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Phytochemical screening, antioxidant activity and toxicity of the orchids *Prosthechea cochleata* and *Prosthechea livida*-A preliminary study

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ABSTRACT

Phytochemical composition, antioxidant, and toxic effects of two orchid *(Prosthechea cochleata* and *Prosthechea livida*) were examined in their different hexane and dichloromethane/methanol extracts of leaves, pseudobulbs, rhizome and roots. Phytochemical screening reveals the presence of alkaloids, coumarins, flavonoids, saponins, steroids and terpenes except for anthracene derivatives in *P. livida* and tannins in both species. Results showed exhibited significant toxicity against brine shrimp with an LC_{50} values ranged from 3 to 54 µg/ml in 24 h. Finally, the results of the antioxidant test in yeast (*Saccharomyces cerevisiae*) showed that CH_2Cl_2:MeOH extract of *P. cochleata* root extract presented the most efficient protective antioxidant percentage (%PA), with 20 µg/mL, displaying a cell viability of 68.58%. In *P. livida* case, rhizome extract presented the most efficient %PA, with 1000 µg/mL, with a cell viability of 65.82%. The results evidenced the toxicity of *Prosthechea cochleata* and *Prosthechea livida* and reveals potential antioxidative of extracts.

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INTRODUCTION

The use of medicinal plants to treat several diseases is a usual practice that is increasing around the world. This knowledge has been used for finding bioactive secondary metabolites, contributing to the development of new drugs. Orchids are a large family of plants with around 25,000 species widespread in the world [1,2]. Orchids are mainly used as ornamental plants. Nevertheless, these plants have played an important role in ancient cultures medicine [3,4]. The research on orchids´ metabolites has been carried out with several species, resulting in the discovering of molecules with a widely chemical activity range [3, 4, 5, 6, 7, 8]. In Mexico, orchids have been mainly used within the industries of regional handmade crafting and gastronomy, and in the celebration of religious rites, as well. Orchids are also used in other industries such as medicine as a component to manufacture narcotics, and for medical purposes[3,9].

The genus *Prosthechea* belongs to the Mesoamerican taxa which is composed of around 90 different species. It is proved that 47 of these species are located in Mexico. Epiphytic plants, occasionally terrestrial, the pseudobulbs are comprised of an internode, single or on group, with 1 to 4 terminal and sessile leaves. These plants present an apical inflorescence, with few or numerous flowers, occasionally showy and with a conspicuous spathe [10]. In recent years, the pharmacological research on *Prosthechea* has increased due to the potential of these orchids mainly related to the antidiabetic activity, making *Prosthechea michuacana* one of the most studied orchids. Anti-diabetic, hypoglycemic, antioxidant, anti-inflammatory, and healing properties have been reported to different organic extracts (*n*-hexane, chloroform, and methanol) and organs of the plant (leaves, pseudobulbs, rhizome, and roots) [11-17]. Furthermore, the hydroalcoholic extract of flowers, leaves, and pseudobulbs of *P. karwinskii* reduced the glycemic and lipidemic levels in Wistar rats with induced metabolic syndrome, which was attributed to the extract antioxidant capacity [18, 19].

In the North of Veracruz Mexico, several species of *Prosthechea* have been reported [20], among them *P. cochleata* and *P. livida* that we can find in abundance in the wild and in cultivation by people from the area. In some cases, they fall from the trees due to the heavy colonies or on the other hand they are knocked down for believing that they are parasitic plants. The present study aimed to evaluate the phytochemical compounds of different extracts and organs of *Prosthechea cochelata* and *P. livida*, followed by of the analysis of toxicity in brine-shrimp assay and the potential antioxidant activity in an induced H_2O_2 yeast model oxidative stress.

MATERIALS AND METHODS

Plant Material

P. cochleata (20° 26´ 46.9" N, 096° 22´ 36.2" W) and *P. livida* (21° 18´ 16.4" N. 097° 51´ 05.4" W) were collected in February 2016, in the North of Veracruz, Mexico. The specimens were identified by José Luis Alanís Méndez (PhD), School of Biological and Agricultural Sciences, Universidad Veracruzana, Tuxpan, Mexico. The voucher specimens were stored at the Universidad Veracruzana herbarium (Tuxpan, Veracruz), under the numbers Ver-Her-223-07-09(008) (*P. cochleata*) and Ver-Her-223-07-09(009) (*P. livida*). The plants were divided into leaves, pseudobulbs, rhizomes, and roots. The material was properly washed, cut, and dried before pulverization in a grinder mill, and stored in amber jars at room temperature.

Plant Extracts

To perform the phytochemical analysis of each plant, the hydroalcoholic extracts were prepared separately using 30 g of the dried plant in 100 mL ethanol 70 %. Subsequently, they were sonicated during 5 min and subsequently placed into a water bath at 80°C for 5 min. Each extract was filtered in a Whatman N° 2 filter paper and stored in amber vials. For the pharmacological evaluations (*In-vivo* antioxidant assay and Brine shrimp cytotoxic assay) of nonpolar and polar metabolites, n-hexane and Dichloromethane:Methanol (CH_2Cl_2:MeOH) (1:1) crude extracts were prepared using 100 g of a dried powder from parts of both plants and 100 mL of the solvent extraction by successive macerations (3x for 72 h, each), first with *n*-hexane and then with the halogen-alcohol solvent. After filtration, extracts were concentrated under a vacuum at 40 °C.

Phytochemical analysis

Classical chemical tests for screening alkaloids, anthracene derivatives, coumarins, flavonoids, saponins, steroids, terpenes, and tannins were performed on the hydroalcoholic extracts using standard procedures reported by Wagner and Bladt [21], Harborne [22], Bruneton [23], Carvajal-Rojas *et al.* [24], Tiwari *et al.* [25], Herbert *et al.* [26], and Rivas-Morales *et al.* [27].

Alkaloids: 2 mL of hydroalcoholic extract was evaporated in three tubes. Add 3 mL of aqueous hydrochloric acid (10%). Heat it for few minutes in a bath water. After that, add 3 drops of Dragendorff, Mayer and Wagner reagents in each one of the tubes. A precipitate with/or turbidity confirm alkaloid presence.

Anthraquinones: 0.20 g of dry plant was suspended in 5 mL of chloroform by 15 min. Then, the chloroform extract was divided in two tubes. In one of them, add 1 mL of aqueous solution of sodium hydroxide (5%). A pink color in the solution show the presence of anthraquinone. In the second tube. Add a methanolic solution of magnesium acetate (0.5%). Red or yellow color in aqueous phase confirms anthraquinones.

Coumarins: Put 2 mL of hydroalcoholic extract in a tube. Place a filter paper impregnated with sodium hydroxide (10%) in the mouth of the tube. Heat it in a bath water (100°C) for 5 minutes. Take and dry the filter paper. Examined under UV light, yellow fluorescence being indicative of the presence of coumarins.

Flavonoids: In 2 mL of hydroalcoholic extract was added some fragments of metallic magnesium. Add a few drops of a hydrochloric acid (10%). After to 15 min, add 1 mL of amyl alcohol. The Amyl alcohol staining (yellow to red) is positive evidence for flavonoids.

Saponins: 5 mL of hydroalcoholic extract was evaporated in a tube. Was Added 5 mL of hot water. Cold the solution and shake, rest by 20 min. The presence of foam in the tube is indicative for the presence of saponins.

Steroids: 5 mL was dried and dissolved in 3 mL of chloroform. Was added 2 mL of a solution of acetic anhydride with 2 drops of concentrated sulfuric acid. A green coloration (sometimes blue, violet, pink or red) will be observed that turns to black with time, indicating the presence of steroids.

Triterpenes: 5 mL of hydroalcoholic extract was evaporated in a tube. Add 2 ml of concentrated sulfuric acid and heated for about 2 min. A blood red-yellow coloration is positive for steroids.

Tannins: 5 mL of hydroalcoholic extract was evaporated in a tube. Then, the extract was dissolved with water and filter. Was added some drops of an aqueous solution of ferric chloride (10%). Blue colorations indicate the possible presence of hydrolysable tannins. Green colorations indicate the possible presence of condensed tannins.

In vivo Antioxidant Assay

This test was carried out using the yeast model reported by Golla and Bhimathati. [28], and followed the methodology described by Salgado *et al.* [29] with minor modifications. The yeast was grown in liquid yeast peptone dextrose (YPD) medium at 28°C to reach an exponential growth phase. The cells inoculum was placed in a 24-wells plates with the *n*-hexane and CH_2Cl_2:MeOH extracts at 1000, 500, 250, 100, 50, and 20 µg/mL to a final volume of 1 mL and DMSO

concentration at 5%, which were incubated for 1 h. The treated cells with the extracts were stressed by addition of 4mM hydrogen peroxide prepared in phosphate buffered saline (PBS) solution. Cell viability was calculated by counting colony forming units (CFU) with a cell dilution of 1:10,000 seeded in YPD solid medium plates and incubated for 48h. DMSO (5%) with/without H_2O_2 4mM were assayed as controls for the antioxidant activity. Experiments were conducted in triplicate and reported as mean of cell percentage of survival, which represents the protective antioxidant activity (PA %). The cell viability after treatment with the extract (% CVE) during 1h and then exposed to H_2O_2 (% CVP) for 2h, following the formula: (100 / % CVE) x % CVP, was used to determine de PA%.

Brine Shrimp Lethality Assay

The toxic activity of the *n*-hexane and CH_2Cl_2:MeOH extracts was checked by the brine shrimp lethality test by Meyer *et al*. [30]. The *n*-hexane and CH_2Cl_2:MeOH extracts were dissolved in DMSO to final concentration of 5 % in 0.9% saline solution. The extracts concentrations at 1000, 500, 250, 100, 50, 25, 5, and 1 µg/mL were evaluated in 96-well microplate in triplicate to a volume of 100 µL in sea water. The negative check 5% DMSO and the positive control of dichromate potassium dilutions were used. In each well 10 nauplii (100 µL) were added, and the covered plate was incubated at 22-29 °C for 24 h. Plates were examined under a binocular microscope and the number of dead (non-motile) nauplii in each well were counted and recorded.

Statistics Analysis

The antioxidant activity assay data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey test for comparing the control and the assayed groups, using STATISTICA Version 7.0. Statistical significance was assumed at the level of 0.05. The results are expressed as mean ± standard deviation (SD). The mean results of brine shrimp mortality against logarithm of concentration were plotted using the Microsoft Excel computer program, which also presented regression equations. The regression equations were used to calculate the LC_{50} value.

RESULTS AND DISCUSSION

The qualitative phytochemical screening of *Prosthechea cochleata* and *Prosthechea livida* showed that the hydroalcoholic extracts were positive for alkaloids, coumarins, flavonoids, saponins, steroids, and triterpenes, excepting for anthraquinones in *P. livida*, and tannins in both extracts (Table 1). The general phytochemical characterization of a plant belonging to this genera is shown in *P. michuacana*, where several constituents as: triterpenoids, stilbenes, phenolics, abietane-type diterpenes from the pseudobulbs[12, 16, 17], and flavonoids in pseudobulbs [15] were found. Despite above-mencionated, in *P. karwinski* several flavonoids have been isolated in pseudobulbs, flowers and leaves [18]. As we can see, the pseudobulbs and leaves of the orchids present a great diversity of chemical compounds that could be related to their main functions, which contain the accumulation of nutrients, response to environmental stimuli, and also play basic roles in plant growth [31].

The extracts of *P. cochleata* and *P. livida* showed toxicity against brine shrimp with an LC_{50} values ranged from 3 to 54 µg/ml in 24 h (Table 2). The toxicity of the extracts was considered by comparison to Meyer *et al*. [30] and Clarkson *et al*. [32] toxicity index where LC_{50} values of 500-1000, 100-500, and 0-100 µg/mL were considered low, medium, and highly toxic, respectively. The values obtained in this work according to Meyer or Clarkson could be considered with high toxicity. The use of brine shrimp lethality assay to determinate the toxicity of plant extracts has been considered as a preliminary test [33]. This method is simple, reproducible and can be adapted to do in tube or in 96-well microplate. Several compounds, extracts or cytotoxic agents are toxic to brine shrimp due to sensitivity of this organism [34] (Sasidharan *et al*. 2008). Coe *et al*. [35] reported that extracts with presence of alkaloids were cytotoxic, causing brine shrimp death at LC_{50} concentrations below 2000 µg/mL, and the extracts without alkaloids the toxicity was due to terpenes.

Table 1: Preliminary phytochemical screening of hydroalcoholic extract of *Prosthechea cochleata* and *P. livida*

Phytochemical tests	*Prosthechea cochleata*				*Prosthechea livida*			
	Leaves	Pseudobulbs	Rhizome	Roots	Leaves	Pseudobulbs	Rhizome	Roots
Alkaloids								
Dragendorff	++	+	+	-	++	+	-	+
Mayer	++	+	+	-	+	++	-	+
Wagner	++	++	+	-	++	++	+	+
Coumarins	+	+	+	+	+	+	-	+
Flavonoids	++	++	-	++	++	++	++	+
Tannins	-	-	-	-	-	-	-	-
Saponins	++	+	-	-	++	-	-	+
Triterpenoids and/or Steroids								
Liebermann-Burchard	+++	++	-	-	++	+	+	++
Salkowski	-	+++	+++	++	+	++	++	+++
Anthracene derivatives Borntrager	-	+	++	+	-	-	-	-

(+++) High concentration of the metabolite; (++) Medium concentration of the metabolite; (+) Minimal presence of the metabolite, (-) There is no presence of the metabolite

Table 2: Toxicity (LC_{50}) of *Prosthechea* extracts in *Artemia salina* nauplii

Specie	*Prosthechea cochleta*		*Prosthechea livida*	
	Hexane	CH_2Cl_2: MeOH	Hexane	CH_2Cl_2: MeOH
Pseudobulbs	13.98 μg/mL	10.06 μg/mL	4.17 μg/mL	10.49 μg/mL
Leaves	10.48 μg/mL	7.49 μg/mL	5.89 μg/mL	19.78 μg/mL
Rhizome	35.88 μg/mL	2.83 μg/mL	16.42 μg/mL	10.58 μg/mL
Roots	54.48 μg/mL	5.13 μg/mL	27.78 μg/mL	9.94 μg/mL
Control Potassium dichromate		18.73 μg/mL		

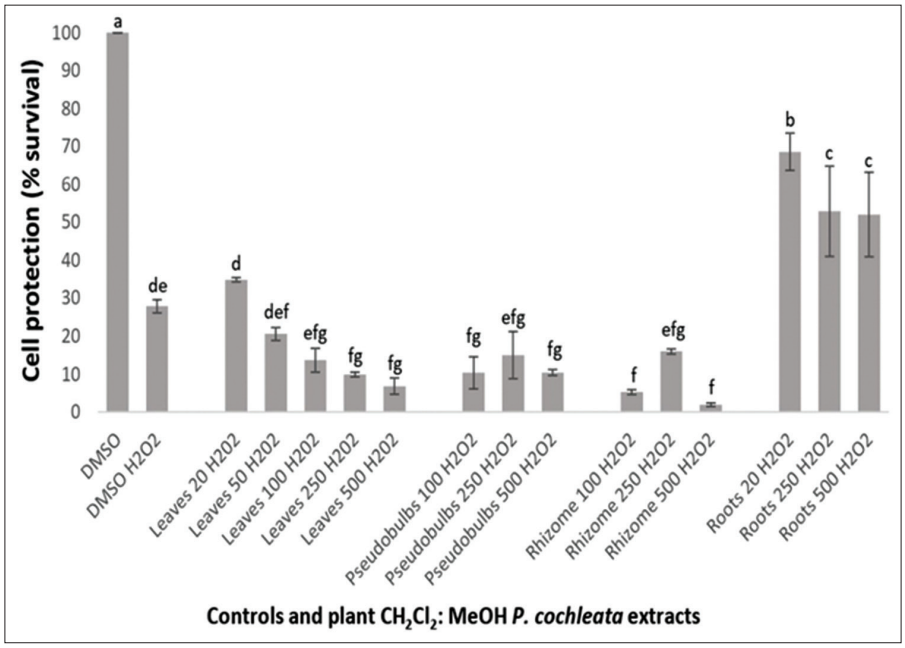

Figure 1: Percentage of yeast cell protection (% survival) towards the H_2O_2 (4mM) oxidative stress, for different concentrations of the leaf, pseudobulb, rhizome and roots CH_2Cl_2:MeOH extracts from *P. cochleata* (μg/mL). DMSO and DMSO+H_2O_2 were used as controls. Different letters indicate significant difference ($p \leq 0.05$)

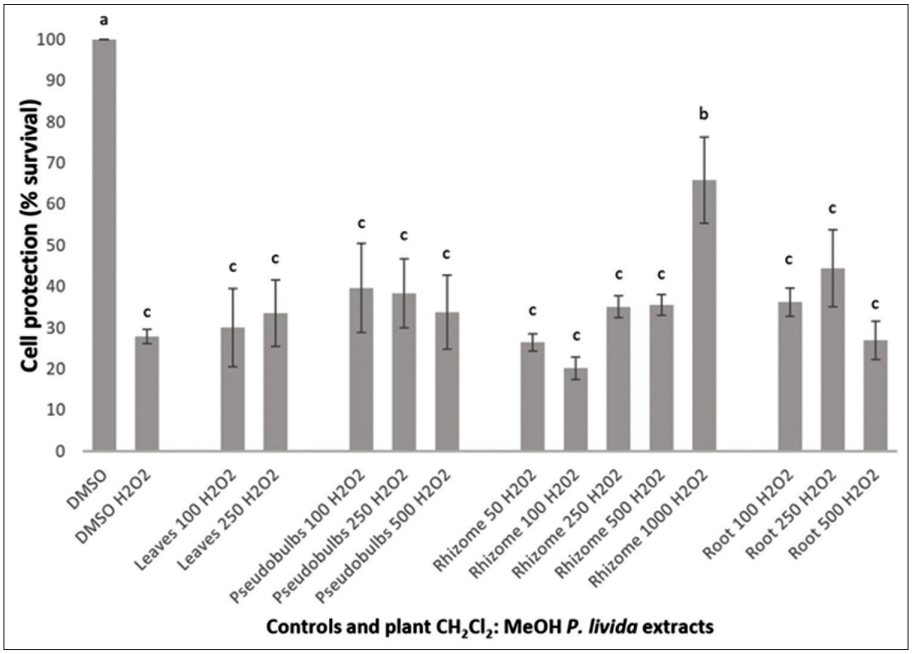

Figure 2: Percentage of yeast cell protection (% survival) towards the H_2O_2 (4mM) oxidative stress, for different concentrations of the leaf, pseudobulb, rhizome and roots CH_2Cl_2:MeOH extracts from *P. livida* (μg/mL). DMSO and DMSO+H_2O_2 were used as controls. Different letters indicate significant difference ($p \leq 0.05$)

In this study, all CH_2Cl_2: MeOH *Prosthechea* extracts were positive for alkaloids and other compounds resulting in the toxicity of this.

The CH_2Cl_2:MeOH extract of *Prosthechea* species showed significant antioxidant activity *in-vivo* by increase the survival percentage of yeast cells against H_2O_2 (figure 1 and 2). In *P. cochleata*, root extract presented the most efficient protective antioxidant percentage (%PA), with 20 µg/mL, displaying a cell viability of 68.58%. In *P. livida* case, rhizome extract presented the most efficient %PA, with 1000 µg/mL, with a cell viability of 65.82%. The antioxidant activity represented by cell viability may be attributed to the presence of flavonoids observed in the phytochemical screening that were identified in roots of *P. cochleata* and rhizome of *P. livida*. In the case of *Prosthechea michuacana* four flavonoids were isolated by Gutierrez-Perez *et al*. [14].

CONCLUSIONS

Further studies will be necessary in order to elucidate the chemical constituents of the organic extracts of *P. cochleata* and *P. livida*, as well as their pharmacological properties of these molecules for a better understanding about the medicinal potential of these plants. In the present research, the results suggest that the extracts of *P. cochleata* and *P. livida* can be used as potential antioxidant on human health as hepatoprotective or anticancer drugs. These extracts also have toxic effects, and it will be necessary to develop further studies to prove their efficacy and safety of its components.

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REFERENCES

1. Dixon KM, Kell SP, Barrett RL, Cribb PJ. Orchid Conservation. Natural History Publications: Kota Kinabalu; 2003.
2. Dressler RL. How many orchid species? Selbyana. 2005;26:155-158.
3. Hossain MM.. Therapeutic orchids: traditional uses and recent advances, an overview. Fitoterapia. 2011;82:102–140.
4. Teoh ES. Medicinal orchids of Asia. New York. Springer International; 2016.
5. Kong JM, Goh NK, Chia LS, Chia TF. Recent advances in traditional plant drugs and orchids. Acta Pharmacologica Sinica, 2003;24(1): 7-21.
6. Acharya KP, Rokaya MB. Medicinal orchids of Nepal: Are they well protected? Our Nature. 2010;8:82-91.
7. Pérez-Gutiérrez RM. Orchids: A review of uses in traditional medicine, its phytochemistry and pharmacology. Journal of Medicinal Plants Research. 2010;4(8):592-638.
8. Marasini R, Joshi S. Antibacterial and antifungal activity of medicinal orchids growing in Nepal. J. Nepal Chem. Soc, 2012;29:104-109.
9. Cruz-García G, Solano-Gomeza R, Lagunez-Rivera L. Documentation of the medicinal knowledge of *Prosthechea karwinskii* in a Mixtec community in Mexico. Brazilian Journal of Pharmacognosy. 2014;24:153-158.
10. García-Cruz J, Sánchez LM, Jiménez R, Solano R. "Familia Orchidaceae: Tribu Epidendreae". Flora del Bajío y de Regiones Adyacentes. 119. Pátzcuaro, Michoacán, México. Instituto de Ecología, A.C. Centro Regional Bajío; 2005.
11. Pérez-Gutiérrez RM. Orchids: A review of uses in traditional medicine, its phytochemistry and pharmacology. Journal of Medicinal Plants Research. 2010;4(8):592-638.
12. Pérez-Gutiérrez RM, Gómez y Gómez Y, Bautista-Ramírez E. Nephroprotective activity of *Prosthechea michuacana* against Cisplatin-induced acute renal failure in rats. Journal of Medicinal Food. 2010; 13(4): 911-916.
13. Pérez-Gutiérrez RM, Neira-González AM, García-Baez E, Lugardo-Diaz S. Studies on the constituents of bulbs of the orchid *Prosthechea michuacana* and antioxidant activity. Chemistry of Natural Compounds. 2010;46(4):554-561.
14. Pérez-Gutiérrez RM, Anaya-Sosa I, Hoyo-Vadillo C, Cruz-Victoria T. Effect of flavonoids from *Prosthechea michuacana* on carbon tetrachloride induced acute hepatotoxicity in mice. Pharmaceutical Biology. 2011;49(11):1121-1127.
15. Pérez-Gutiérrez RM, Hoyo-Vadillo C. Anti-diabetic activity of an hexane extract of *Prosthechea michuacana* in streptozotocin-induced diabetic rats. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas. 2011;10(6):570-580.
16. Pérez-Gutiérrez RM. Evaluation of the hipoglycemy and hypolipidemic effects of triterpenoids from *Prosthechea michuacana* in streptozotocin-induced Type 2 Diabetic mice. Pharmacologia, 2013;4:170-179.
17. Pérez-Gutiérrez RM. Inhibition of advanced glycation end products formation by stilbene and phenanthrene derivatives from *Prosthechea michuacana in vitro* and *in vivo*. Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas. 2013;12(1):69-80.
18. Rojas-Olivos A, Solano-Gómez R, Alexander-Aguilera A, Jiménez-Estrada M, Zilli-Hernández S, Lagunez-Rivera L. Effect of *Prosthechea karwinskii* (Orchidaceae) on obesity and dyslipidemia in Wistar rats. Alexandria Journal of Medicine. 2017;53(4):311-315.
19. Rojas-Olivos A, Solano-Gómez R, Jiménez-Estrada M, Silva-Valeriano Y, Lagunez-Rivera L. (2014). Antioxidant capacity of *Prosthechea karwinskii* (Orchidaceae) extracts obtained by sonication. Journal of Chemical, Biological and Physical Sciences. 2014;4(3):2249-1929.
20. Alanís-Mendez JL. Spatial distribution of wild orchids of the Ecological Reserve "Sierra de Otontepec" Veracruz, Mexico. PhD Thesis in Environmental Sciences to Xalapa University; 2012.
21. Wagner H, Bladt S. Plant Drug Analysis. A Thin Layer Chromatography Atlas.2nd ed. Berlin Heidelberg: Springer-Verlag; 1996.
22. Harborne AJ. Phytochemical Methods A Guide to Modern Techniques of Plant Analysis. 3rd ed. Netherlands: Springer; 1998.
23. Bruneton J. Pharmacognosy, phytochemistry, medicinal plants.2nd ed. Spain: Acribia editorial; 2001.
24. Carvajal-Rojas L, Hata-Uribe Y, Sierra-Martinez N, Rueda-Niño D. Preliminary phytochemical analysis of Cupatá (*Strychnos schultesiana* krukoff) stems and seeds, Revista Colombia Forestal, 2009;12: 161-170.
25. Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H. Phytochemical screening and extraction: A review. International Pharmaceutica Sciencia, 2011;1(1):98-106.
26. Herbert-Doctor LA, Saavedra-Aguilar M, Villarreal-Ortega ML, Cardoso-Taketa A, Vite-Vallejo O. Insecticidal and nematicidal effects of *Agave tequilana* juice against *Bemisia tabaci* and *Panagrellus redivivus*. Southwestern Entomologist, 2016;41(1): 27-40.
27. Rivas-Morales C, Oranday-Cárdenas MA, Verde-Star MJ. Research in plants of medical importance. UANL-México: Omnia Publisher; 2016.
28. Golla U, Bhimathati SSR. Evaluation of antioxidant and DNA damage protection activity of the hydroalcoholic extract of *Desmostachya bipinnata* L. Stapf. The Scientific World Journal, 2014;2014:1-8.
29. Salgado N, Núñez-Aragón PN, Acevedo M, Corona L, Rodríguez V, Mesnard F, Fliniaux MA., Molinié R, Marcelo P, Villarrea ML, Cardoso-Taketa AT. Radical scavenging, antioxidant, and cytotoxic activities of the methanolic extracts from different organs of *Ternstroemia pringlei*. Pak. J. Pharm. Sci, 2015; 28(6):2279-2284.
30. Meyer B, Ferrigni N, Putnam J, Jacobsen L, Nichols D, McLaughlin J. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Medica,1982;45(5):31–34.
31. Sato F. Plant Secondary Metabolism. eLS. Ltd, Chichester: John Wiley & Sons; 2014.

32. Clarkson C, Maharaj VJ, Crouch NR., Olwen MG, Pillay P, Matsabisa MG, Bhagwandin N, Smith PJ, Folb PI. *In-vitro* antiplasmodial activity of medicinal plants native to or naturalized in South Africa. Journal of Ethnopharmacology. 2004;92:177–191.
33. Opler A, Mizell R, Robert A, Cervantes-Cervantes M, Kincaid D, Kennelly EJ. Use of a brine shrimp assay to study herbal teas in the classroom. The American Biology Teacher. 2002;64(8):596-604.
34. Sasidharan S, Darah I, Jain K. *In vivo* and *In vitro* toxicity study of *Gracilaria changii*. Pharmaceutical Biology. 2008;46(6):413-417.
35. Coe FG, Parikh DM, Johnson CA. Alkaloid presence and brine shrimp (*Artemia salina*) bioassay of medicinal species of eastern Nicaragua. Pharmaceutical Biology. 2010;48(4):439–445.