Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **28** (6): 863-8 (2009)

Original Article

Received: December 8, 2008
Accepted: July 26, 2009

Antimicrobial Activities of Ethanol Extract and Coumestans from *Eclipta alba* (L.) Hassk (Asteraceae)

Vanessa A. LENZA [1 #], Lucas J.F. MOREL [1#], Juliana S. COPPEDE [1], Vanessa C. FERNANDES [1], Nilce M. MARTINEZ-ROSSI [2], Suzelei C. FRANÇA [1], René O. BELEBONI [1], Paulo S. PEREIRA [1] & Ana L. FACHIN [1*]

[1] *Unidade de Biotecnologia, Universidade de Ribeirão Preto, Av. Costábile Romano, 2201, 14096-900, Ribeirão Preto, São Paulo, Brasil*

[2] *Departamento de Genética, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes, 3900, 14049-900 Ribeirão Preto, São Paulo, Brasil*

SUMMARY. Ethanol extract and fractions from aerial parts of *Eclipta alba* (L.) Hassk (Asteraceae) were screened for the antibacterial and antifungal activities against different species of human pathogenic bacterial ATCC, antibiotic-resistant clinical isolates and strains of the dermatophyte *Trichophyton rubrum* (wild and mutant for *Tru*MDR2 gene) using a microdilution method. Demethylwedelolactone/wedelolactone (DWL/WL) and only wedelolactone (WL), both in a high homogeneity degree, were efficient to inhibit the ATCC strains of *Staphylococus aureus* (Minimal Inhibitory Concentration MIC = 75 μg/mL), *Staphylococcus epidemidis* (MIC = 125 μg/mL) and *Escherichia coli* (MIC = 125 μg/mL) as well as antibiotic-resistant clinical isolates of *Enterococcus* spp (MIC = 250 μg/mL) and *S. aureus* (MIC = 125 μg/mL). Ethanol extract was more effective than the purified fractions against *Trichophyton rubrum* strains (MIC = 125 μg/mL), suggesting that anti-fungal activity is not only related to demethylwedelolactone and wedelolactone, but also to a synergistic action between these coumestans and other compounds found in that extract. Thus, this work suggests that *E. alba* possesses a significant antimicrobial activity, including that against multi-drug resistant microorganisms, which could be of relevance for the treatment of infectious diseases.

INTRODUCTION

Currently, it has been observed a significant growth in the emergence and dissemination of resistant microorganisms to antimicrobial drugs and consequently a decrease in the efficacy of available therapeutic approaches [1]. For example, methicillin-resistant *Staphylococcus aureus* strains become endemic in hospitals and intensive care units, while, only vancomicine appears to be an effective antibiotic for patients infected with this bacteria [2].

Human fungal infections vary from superficial to invasive or disseminated, and have drastically increased in the last few decades [3]. Mycosis treatment has been based on the same strategy for antibacterial therapy; however, only antifungal drugs are available compared to the number of antibacterial ones [4]. Fungal infections in immunocompromised individuals have greatly increased in recent years, promoting the natural selections of pathogens that so far were not considered etiological agents of systemic mycosis, constituting a serious public healthcare problem [5]. Among the onychomycosis associated with AIDS, 85% of them are caused by *T. rubrum,* which has been characterized as clinically aggressive and by a high frequency of unusual presentation and resistance to the conventional therapy [6].

Antifungal and antibacterial agents are often from synthetic sources. Regarding the number of drug resistant microorganisms and technical disturbances actually observed on some clinical therapeutics, such as, drug low potency, side effects and costs, the development of new medicines with different chemical moeties,

KEY WORDS: Antimicrobial activity, Demethylwedelolactone, *Eclipta alba,* Wedelolactone.

* Author to whom correspondence should be addressed. *E-mail:* asaltoratto@unaerp.br
\# Authors with equal contribution.

mode of actions may be considered a relevant strategy [7]. Thus, the search for new antimicrobial agents from medicinal plants could be placed as an interesting approach, particularly in developing countries where the frequency of infectious diseases is increasing in a fast ratio [8,9].

Eclipta alba (L.) Hassk (Asteraceae) (syn: *E. prostrata*) is an herb commonly found in the Amazon region and popularly known as "agrião-do-brejo" or "erva-botão". *E. alba* aerial parts are commonly used in Brazilian folk medicine against snake bite [10]. Recent reports indicate that *E. alba* have immunostimulator and hepatoprotective properties [11]. It has also been noticed that *E. alba* possesses anti-inflammatory, analgesic, anti-aging, hair growth promoting and nervine tonic activities [12-14]. Moreover, *E. alba* has been related to the treatment of several chronic skin diseases [15]. *E. alba* phytochemical profile is characterized by the presence of eclalbatin, alpha-amyrin, ursolic acid, oleanolic acid [16], ecliptasaponin, daucosterol, stigmasterol-3-O-glucoside [17] and coumestans (demethylwedolactone and wedelolactone) [18].

The aim of this work was to evaluate the antimicrobial activity of *E. alba* ethanol extract and active fractions against *Trichophyton rubrum* strains, bacterial ATCC strains and antibiotic resistant clinical isolates.

MATERIAL AND METHODS

Plant material

Aerial parts of *E. alba* were collected in Ribeirão Preto, SP, Brazil (GPS -21° 11' 55,2"-47° 46' 42,1"). Voucher specimens (N HPMU 058) were deposited at the Herbarium of Ribeirão Preto University, São Paulo, Brazil.

Ethanol extracts preparations and active compounds purification

Aerial parts of *E. alba* (2200 g) were dried, pulverized and then macerated in ethanol 95% (5:1; w/v) for 24 h at 25 °C. After filtration, the material was concentrated in a rotatory evaporator and lyophilized, yielding 295.8 g of the ethanolic dried extract. Ethanol extract (100 g) was dissolved in 500 mL of methanol: pure water (1:1; v/v) and submitted to extraction with n-hexane (500 mL) for three times. The Water dissoluble fraction (WF) was concentrated in a rotor evaporator until the water volume had been reduced by 50% and then submitted to extraction with ethyl acetate (300 mL) for three times. The hexane fraction (HF) and ethyl- acetate fraction (EAF) were evaporated and the water dissoluble fraction (WF) lyophilized.

The EAF (5 g) was submitted to purification on a Sephadex® LH20 chromatographic column (3.3 x 120 cm) using methanol as mobile phase. After TLC analysis the fractions were selectively grouped. The TLC conditions were: silica gel plate (10 x 10 cm), chloroform:methanol (8:2; v/v) as mobile phase and iodine vapors and sulphuric vanillin as developers. Coumestans fractions were applied on a RP18 HPLC column (Supelcosil™ Sigma-Aldrich, St. Louis, MO, USA. RP-18, 250 x 10 mm-i.d.), yielding wedelolactone (32.5 mg; R_t = 70-80 min) and demethylwedelolactone (16.0 mg; Rt = 50-62 min). Detection was at 210 and 350 nm. A two-solvent gradient system of water (A) and methanol (B) was used. The gradient program consisted of four time periods: (1) 0-100 min, 0-60% B, (2) 100-110 min, 60% B, (3) 110-112 min, 60-0% B and (4) 112-120 min, 0% B. The flow-rate was 2.0 mL/min, the sample injection volume was 0.8 ml (100 mg/mL). Chromatographic fractions containing Demethylwedelolactone and Wedelolactone (DWL/WL) and only Wedelolactone (WL), both in a high homogeneity degree, were identified by means of HPLC profile, and UV and NMR (^{1}H and ^{13}C) spectra. In order to perform the microbiological assay, the ethanol extract and fractions were dissolved in 10% DMSO (dimethyl sulfoxide) in pure water.

Fungi strains

A clinical *T. rubrum* isolate (ATCC-MYA3108) was obtained from a patient admitted to the University Hospital of Ribeirão Preto, SP, Brazil. The mutant strain *Tru*MDR2 was obtained from disruption of the *Tru*MDR2 gene from isolate MYA3108 [19]. Standard protocols for maintenance of both fungal strains were previously described by Fachin *et al.* [19,20].

Antifungal assay

Susceptibility of MYA3108 and *Tru*MDR2 mutant strains was tested by assessing the Minimal Inhibitory Concentration (MIC) of different concentrations of ethanol extract and fractions according to Clinical and Laboratory Standards Institute [21]. Colonies were obtained by growing the strains on Sabouraud plates at 28 °C for 15 days and were harvested by sterile scraping, mixed with sterile saline next filtered through a glass wool. The filtrate was transferred to a sterile tube and adjusted spectrophotometrically at a wavelength of 530 nm ranging from 70 to 75% transmittance. The conidial suspensions were diluted 1:50 (v/v) in RPMI 1640 medium (Sigma Chemical, St Louis, MO) buffered with 4-mor-

pholinepropanesulfonic acid (MOPS), which corresponded to twice the density needed for the test of approximately 3.10^5–5.10^5 CFU/mL. Each micro dilution well containing different concentrations of ethanol extract or fractions in RPMI was inoculated with the diluted conidial suspensions in a final volume of 200 µL. Solvent controls containing 10% DMSO, sterility and growth controls were included for each assay. 96 well plate were incubated at 28 °C and MICs were recorded after 7 days of incubation. The MIC 100 was defined as the lowest concentration at the fraction which resulted in total inhibition. Assays were carried out in three independent experiments performed in triplicate. Fluconazole (0.075 mg/mL) was used as positive control.

Bacterial Strains

The bacterial strains used in the assay were: *Staphylococcus aureus* (ATCC 6538), *S. epidermidis* (ATCC 2228), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853).

Antibiotic resistant isolates

Antibiotic resistant isolates of *S. aureus, Enterococcus* spp, *E. coli, Citrobacter sp, Klebsiella oxytoca, Proteus mirabilis* and *Klebsiella pneumoniae* were collected from urine samples of patients admitted to the Laboratory for Biomedical Analysis at Ribeirão Preto University. The identification was achieved through standard procedures [22] and the antibiogram obtained through the Kirby-Bauer method using antibiotic disks in a concentration of 5 to 30 µg/mL in Mueller Hinton medium [23].

Antibacterial assay

Antimicrobial activity against ATCC strains and antibiotic resistant strains were evaluated according to the microdilution method in a BHI (Brain Heart Infusion) medium [24]. Briefly, each microdilution well containing different concentrations of ethanol extract or fractions was inoculated with the bacterial suspension in a final concentration of 10^5 CFU/mL. Solvent controls containing 10% DMSO, sterility and growth controls were included for each assay. Microtitre trays were incubated at 37 °C and the MIC 100, as defined in the previous section, was recorded after 24 h of incubation. The assays were carried out in three independent experiments performed in triplicate. Ampicilin was used as positive control.

RESULTS AND DISCUSSION

The antibacterial activities of *E. alba* ethanol extract and fractions against ATCC strains are shown in Table 1. DWL/WL and WL fractions, were able to inhibit gram positive as well as gram negative bacteria. The DWL/WL fraction presented MICs of 75, 125 and 125 µg/mL, for *S. aureus, S. epidermidis* and *E. coli,* respectively. The growth of *P. aeruginosa* was completely inhibited by the WL fraction (MIC = 250 µg/mL). It should be emphasized, that infections caused by *P. aeruginosa* are among the most difficult to treat with conventional antibiotics [25]. Usually, the natural antibiotics from plants are more efficient to inhibit gram-positive bacteria than the gram-negative ones [26]. Unlike gram-positive bacteria, the lipopolysaccharide layer along with proteins and phospholipids are the major components in the outer surface of gram-negative bacteria. Compounds access the peptidoglycan layer of the cell wall is hindered by the outer lipopolysaccharide layer [27]. Our results indicate that *E. alba* is a source of active coumestans against infections caused by two types of bacteria.

Bacteria have the genetic ability to transmit and acquire resistance to drugs that are employed as therapeutic agents [28]. Such fact cause

Strains	**EE** [a]	**WF** [a]	**EAF** [a]	**HF** [a]	**DWL/WL** [b]	**WL** [b]	**Ampicilin** *
S. aureus	2500	5000	1250	5000	75	250	19
S. epidermidis	1250	5000	625	1250	125	500	156
E. coli	5000	5000	5000	5000	125	>1000	19
P. aeruginosa	5000	5000	2500	5000	>1000	250	78

Table 1. Minimal Inhibitory Concentration (µg/mL) of ethanol extract and fractions from *E. alba* against ATCC bacterial strains. **EE**: Ethanol extract; **WF**: Water dissoluble fraction; **EAF**: Ethyl acetate fraction (Rich in coumestans); **HF**: Hexane fraction; **DWL/WL**: Demethylwedelolactone and wedelolactone fraction in a high homogeneity degree; **WL**: Wedelolactone fraction in a high homogeneity degree; [a] : Range of concentration 19.5- 5000 µg /mL; [b] : Range of concentration 19.5- 1000 µg /mL; *Ampicilin (19-2500 µg /mL): Reference standard.

Samples	**Resistant**	**EE** [a]	**WF** [a]	**EAF** [a]	**HF** [a]	**DWL/WL** [b]	**WL** [b]
Enterococcus spp	T, CP, G, NO, NT	312	>5000	78	>5000	NT	250
E. coli	A,TS	>5000	>5000	>5000	>5000	NT	>500
K. oxytoca	A	>5000	>5000	>5000	>5000	NT	>500
Citrobacter sp	A	>5000	>5000	>5000	>5000	NT	>500
K. pneumoniae	A, AC, T, TS,	>5000	>5000	>5000	>5000	NT	>500
S. aureus	A, P	78	1250	78	156	NT	125
Proteus mirabilis	NT	>5000	>5000	>5000	>5000	NT	>500

Table 2. Minimal Inhibitory Concentration (µg/mL) of ethanol extract and fractions from *E. alba* against antibiotic resistant clinical isolates. **T**: Tetracycline; **CP**: Ciprofloxacin; **A**: Ampicilin; **G**: Gentamicin; **NO**: Norfloxacin; **NT**: Nitrofurantoin; **TS**: Association trimetoprin/ sulfametazone; **P**: Penicilin; **AC**: , Nalidixic acid. **EE**: Ethanol extract; **WF**: Water dissoluble fraction; **EAF**: Ethyl acetate fraction (Rich in coumestans); **HF**: Hexane fraction; **DWL/WL**: Demethylwedelolactone and wedelolactone fraction in a high homogeneity degree; **WL**: Wedelolactone fraction in a high homogeneity degree; [a] : Range of concentration 19.5-5000 µg /mL; [b]: Range of concentration 19.5-500 µg /mL; **NT**: not tested.

***T. rubrum* strains**	**EE** [a]	**WF** [a]	**EAF** [a]	**HF** [a]	**DWL/WL** [b]	**WL** [b]	**Fluconazole***
MYA-3108	125	1250	2500	1250	>500	500	75
Δ*Tru*MDR2	125	625	2500	1250	500	500	75

Table 3: Minimal Inhibitory Concentration (µg/mL) of ethanol extract and fractions from *E. alba* against *T. rubrum* strains. **EE**: Ethanol extract; **WF**: Water dissoluble fraction; **EAF**: Ethyl acetate fraction (Rich in coumestans); **HF**: Hexane fraction; **DWL/WL**: Demethylwedelolactone and wedelolactone fraction in a high homogeneity degree; **WL**: Wedelolactone fraction in a high homogeneity degree. [a] : Range of concentration 19.5-5000 µg /mL; [b] : Range of concentration 19.5-500 µg /mL; * Fluconazole: Reference standard.

concerns, due to the large number of hospitalized immunocompromised patients who may be infected by new bacterial strains multi-resistant to antibiotics. Regarding the antibiotic resistant isolates, it was noted that the *Enterococcus* spp and *S. aureus* bacterial strains were more sensitive to the EAF (rich in coumestans) and notably to the WL fraction (Table 2). Finally, the ethanol extract, EA and WL fractions showed the most promising results for the two species of resistant clinical isolates *Enterococcus spp* and *S. aureus* (Table 2).

Regarding the antifungal testing, the *E. alba* ethanol extract showed a MIC value of 125 µg/mL for both *T. rubrum* strains. For cumestans fractions a very similar profile of fungal sensitivity was observed. DWL/WL fraction presented a MIC value higher than 500 µg/mL for the MYA-3108 strain and of 500 µg/mL for mutant one, while, WL fraction presented a MIC value of 500 µg/mL for both strains (Table 3). Hence, the ethanol extract was more effective than DWL/WL and WL fractions against the *T. rubrum* strains, suggesting that anti-fungal activity is not only related to demethylwedelolactone and wedelolactone, but also to a synergistic action of between coumestans and other compounds found in that extract.

Unlike observed for DWL/WL and WL fractions, the greater sensitivity of the *T. rubrum* mutant strain to WF fraction in relation to wild one is indicative that the *Tru*MDR2 gene, ruptured in the mutant strain, may be involved in the transportation of active compounds found in this fraction. *T. rubrum,* a filamentous fungus, has been involved in a huge number of clinical dermatophytosis cases, being characterized mainly by its resistance to conventional antifungal treatments and by acting invasively in immunocompromised patients [29-31]. Aiming to clarify the mechanisms of resistance to antifungals in *T. rubrum,* Fachin *et al.* (2006) [19] cloned and sequenced the *Tru*MDR2 gene which codifies an ABC type transporter in this dermatophyte. The rupture of the *Tru*MDR2 gene demonstrated that the mutant became more sensitive to terbinafine, 4NQO and ethidium bromide, suggesting that this transporter acts in the

modulation of susceptibility to drugs in *T. rubrum*. Such mutant became an important experimental model for the research of multiple resistance drugs *in vitro,* which should aid in the development of compounds that may be efficient when the usual therapy is not effective.

The use of medicinal plants and phytochemicals can be of great significance in the antimicrobial therapy. In the last decades, a number of studies using medicinal plants have been conducted to prove such efficiency, chiefly due to the bacterial and fungal multi-resistance to drugs [32-36]. Indeed, Voravuthikunchai & Mitchell [37] screened twenty four medicinal plants extracts widely used in Thailand showing that they can be used as possible sources of drugs to treat multiple antibiotic resistant *Helicobacter pylori*. Antifungal activity of Brazilian medicinal plants used to treat common mycoses was evaluated by Cruz *et al*. [38]. The results showed that the aqueous extract of *Ziziphus joazeiro* and *Caesalpinea pyramidalis* presented a significant antifungal activity against *T. rubrum, Candida guilliermondii, Candida albicans* and *Cryptococcus neoformans*.

The great interest for the search of new natural antimicrobial agents has been motivated by the proposal that natural products could be in most cases advantageous when compared to synthetic ones. In fact, the search for new antimicrobial of differing chemical natures and natural sources could represent an improvement of potency, safety, and costs in relation to synthetic agents presently employed against infectious diseases [39]. Moreover, natural products, containing different active compounds, present complementary mode of actions, particularly when used in a phytocomplex form. It is therefore reasonable to assume that natural antimicrobials are less disposed to cause the development of microorganism resistance when compared to the synthetic ones [40].

CONCLUSION

This work demonstrated that *E. alba* possesses a pronounced antimicrobial activity, especially for multi-drug resistant strains of bacterial clinical isolates and for the dermatophyte *T. rubrum,* which could be of relevance for the treatment of infectious diseases.

Acknowledments. The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for financial supporting and to Adriana Pelegrino Pinho Ramos and Janine de Castilho for technical assistance.

REFERENCES

1. Smith, A. & J. Bagg (1998) *Dent. Update* **25**: 203-8.
2. Burton, M.J., P. Shah & E. Swiatlo (2008) *Am. J. Med. Sci.* **335**: 327-8.
3. Smith, K. J., M.Welsh & H. Skelton (2001) *Br. J. Dermatol.* **145**: 344–8.
4. Osborne, C. S., I. Leitner, B. Favre, & N. S. Ryder (2005) *Antimicrob. Agents. Chemother.* **49**: 2840–4.
5. Vilela, R., F.O. Teixeira, L.Vilela & L. Mendoza (2007) *Mycopathologia* **163**: 203-5.
6. Surjushe, A., R. Kamath, C., Oberai, M. Thakre, D. Saple, S. Dharmshale & A Gohil (2007) *Indian J. Dermatol. Venereol. Leprol.* **73**: 391-401.
7. Fortes, T.O., D.S. Alviano, G. Tupinamba, T.S Padr´On, A.R. Antoniolli, C.S Alviano & L. Seldin (2008) *Microbiol. Res.* **163**: 200-7.
8. Abebe, D., A. Debella & K. Urga (2003) *Br. Med. J.* **317**: 647-50.
9. Mackeen, M.M., A.M. Ali, N.H. Lajis, K. Kawazu, Z. Hassan, M. Amran, M. Habsah, L.Y. Mooi & S.M. Mohamed (2000) *J. Ethnopharmacol.* **72**: 395-402.
10. Mors, W.B., M.C. Nascimento, J.P. Parente, M.H. Silva, P.O. Melo & G.Suarez-Kurtz (1989) *Toxicon* **27**: 1003–9.
11. Christybapita, D., M. Divyagnaneswari & R. Dinakaran Michael (2007) *Fish Shellfish Immunol.* **23**: 840-52.
12. Vaidya, A.B. (1997) *Indian J. Pharmacol* **29**: 340-3.
13. Uniyal, R.C., S. Sandhu, J.K. Chandok (1998) *"Herbology"*. in: "The Ayurvedic Encyclopedia. Sri Satguru Publications", India, pp. 77.
14. Leal, L.K., A.A. Ferreira, G.A. Bezerra, F.J. Matos & G.S. Viana (2000) *J. Ethnopharmacol.* **70**: 151-9.
15. Sawant, M., J.C. Isaac & S. Narayanan (2004) *Phytother. Res.* **18**: 111-3.
16. Upadhyay, R.K., M.B. Pandey, R.N. Jha & V.B. Pandey (2001) *J. Asian Nat. Prod. Res.* **3**: 213-7.
17. Zhang, M. & Y. Chen (1996) *Zhongguo Zhong Yao Za Zhi* **21**: 480-510.
18. Wagner H, B., Geyer, Y. Kiso, H. Hikino & G.S. Rao (1986) *Planta Med.* **5**: 370-4.
19. Fachin, A.L., M.S., Ferreira-Nozawa, W. Jr. Maccheroni & Martinez-Rossi, N.M. (2006) *J. Med. Microbiol.* **55**: 1093-9.
20. Fachin, A.L., E.P. Contel & N.M. Martinez-Rossi (2001) *Med. Mycol.* **39**: 129-33.
21. Clinical And Laboratory Standards Institute (2002) *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi*. CLSI document M38-A. NCCLS, 940. West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA 22. Murray, P.R. (1999) Manual of Clinical Microbiology. Washington: Ellen Jo Baron.

23. Clinical And Laboratory Standards Institute (2000) *Performance standards for antimicrobial disk susceptibility tests*. Wayne, Pennsylvania: National Committee for Clinical Laboratory Standards. NCCLS Document M2-A7.
24. Clinical And Laboratory Standards Institute (2006) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically. Approved standard M7-A7. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA.
25. Levison, W.E. & E. Jawetz (1992) *Medical Microbiology and Immunology,* 2nd ed. Appleton and Lange, New York.
26. Kumar, V.P., N.S Chauhan, H. Padh, & Rajani, M. (2006) *J. of Ethnopharmacol.* **107**: 182-188.
27. Burn, P. (1988) *Trends Biochem. Sci.* **13**: 79-83.
28. Cohen, M.L. (1992) *Science* **257**: 1050-5.
29. Pielop, J. & T. Rosen (2001) *J. Am. Acad. Dermatol.* **44**: 864-70.
30. Kwon, K.S., H.S. Jang, H.S. Son, C.K. Oh, Y.W. Kwon, K.H. Kim & S.B. Suh (2004) *J. Dermatol.* **10**: 839-43.
31. Siqueira, E.R., J.C. Ferreira, C.M.L. Maffei & R.C. Candido (2006) *Rev. Soc. Bras. Med. Trop.* **39**: 269-71.
32. Batista, O., A Duarte, J Nascimento, M.F Simões, M.C. De La Torre & B. Rodriguez (1994) *J. Nat. Prod.* **57**: 858-61.
33. Primo, V., M. Rovera, S. Zanon, M. Oliva, M. Demo, J. Daghero & L. Sabini (2001) *Rev. Argent. Microbiol.* **33**: 113-7.
34. Holetz, F.B., G.L. Pessini & N.R. Sanches (2002) *Mem. Inst. Oswaldo Cruz* **97**: 1027-31.
35. Pretto, J.B. V, Cechinel-Filho, V.F., Noldin, M.R. Sartori, D.E. Isaias, & A.B. Cruz (2004) *Z. Naturforsh.* **59**: 657-62.
36. Lee, S., J. Han, G. Lee, M. Park, I. Choi, K. Na & E. Jeung (2007) *Biol. Pharm. Bull.* **30**: 184-8.
37. Voravuthikunchai, S.P. & H. Mitchell (2008) *J. Health Sci.* **54**: 81-8.
38. Cruz, M.C.S.; P.O. Santos, A.M. Barbosa Jr, D.L.F.M. De Melo, C.S. Alviano, A.R. Antoniolli; D.S.B. Alviano & R.C. Trindade (2007) *J. Ethnopharmacol.* **111**: 409-12.
39. Tripathi, P. & N. K. Dubey (2004) *Postharvest Biol. Technol.* **32**: 235-45
40. Cowan, M. M. (1999) *Clin. Microbiol. Rev.* **12**: 564-82.