

Full Length Research Paper

Anti-HIV-1 activity in human primary cells and Anti-HIV-1 RT inhibitory activity of extracts from the red seaweed *Acanthophora spicifera*

Caio Cesar Richter Nogueira^{1,2}, Izabel Christina Nunes de Palmer Paixão², Claudio Cesar Cirne-Santos³, Paulo Roberto Soares Stephens⁴, Roberto Campos Villaça¹, Helena de Souza Pereira² and Valéria Laneuville Teixeira^{1*}

¹Departamento de Biologia Marinha, Instituto de Biologia, Universidade Federal Fluminense, P. O. Box 100.644, 24001-970, Niterói, RJ, Brazil.

²Departamento de Biologia Celular e Molecular, Instituto de Biologia, Universidade Federal Fluminense, 24020-141, Niterói, RJ, Brazil.

³Laboratório de Imunologia Clínica, Instituto Oswaldo Cruz, FIOCRUZ, 21.040-900, Rio de Janeiro, RJ, Brazil.

⁴Laboratório de Inovações em Terapias, Ensino e Bioprodutos, Instituto Oswaldo Cruz, FIOCRUZ, 21.040-900, Rio de Janeiro, RJ, Brazil.

Received 22 July, 2016; Accepted 6 September, 2016

First generation drugs such as zidovudine have been extensively used in clinical practice, resulting in the development of HIV resistance to these nucleoside analogs. Several studies have demonstrated the effective anti-HIV activity of natural products derived from seaweeds, suggesting promising sources of substances for the development of novel antiviral drugs. In this paper, the antiviral effect of extracts from the red seaweed *Acanthophora spicifera* on HIV-1 replication was evaluated *in vitro*. Peripheral blood mononuclear cells obtained using the Ficoll-Hypaque gradient were used for cytotoxicity and antiviral activity testing. The dichloromethane extracts, ethyl acetate, acetone, and methanol were found to have CC₅₀ values of 31±7.4, 45±11, 38±3.5, and 179±25 µg/mL, respectively. With the control, the extract prepared in ethyl acetate inhibited approximately 60% of the viral load, which is the best result among the extracts. This same extract showed an IC₅₀ value of 33.17±4.84 µg/mL for the reverse transcriptase. The EtOAc extract from *A. spicifera* showed to be an efficient HIV antiviral due to its phenolic compounds, as evaluated by nuclear magnetic resonance.

Key words: Marine natural products, red seaweed, *Acanthophora spicifera*, HIV-1, Antiviral activity, Anti-HIV-1 RT.

INTRODUCTION

Since the discovery of the human immunodeficiency virus, many drugs have been developed in an attempt to

inhibit its replication. However, HIV is resistant to treatment with known drugs (Kuritzkes, 2007; Hirsch et

*Corresponding author. E-mail: valerialaneuville@gmail.com Tel: +55 (21) 2629-2296.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

al., 2008; Manhanzva et al., 2015). This is due to the high mutation rate of HIV and does not have an effective mechanism for error correction during replication. One of the strategies adopted was the combination of two or more drugs known as Highly Active Antiretroviral Therapy (HAART) (Marrazzo et al., 2014). Such treatment may reduce viral load to undetectable levels in the blood and provide long-lasting clinical benefit. However, some patients do not respond to this treatment, making the search for new molecules with anti-HIV activity an urgent need. Seaweeds are a source of many bioactive compounds. Several extracts, fractions, and natural products isolated from seaweeds have demonstrated effective anti-HIV activity (Vo and Kim, 2010), making it an interesting base from which to develop new medicines. Of the seaweeds, red seaweeds produce natural products such as acetogenins (Gutiérrez-Cepeda et al., 2011), sesquiterpenes (Chen et al., 2016), monoterpenes (Silva et al., 2015), bromophenols (Poplewell and Northcote, 2009), and sulfated polysaccharides (Coura, 2012) that can be used for anti-HIV drug development. *Acanthophora spicifera* (Rhodophyta) is an excellent model for studies of biological activity in Brazil because it has natural banks on the coast of Rio de Janeiro, is easily identified (Perrone et al., 2006), has an experimental field cultivation described in the literature (Kaliaperumal et al., 1986), and is a part of the food chain to other species, indicating a low toxicity (Cruz-Rivera and Villareal, 2005). Furthermore, fractions rich in sulfated polysaccharides from the red seaweed *Acanthophora* are an effective antiviral against HSV-1 and HSV-2 strains (Duarte et al., 2004). Therefore, the objective of this study was to evaluate the antiviral effect of extracts from *A. spicifera* regarding HIV-1 replication in human primary cells and their ability to inhibit the enzyme reverse transcriptase.

MATERIALS AND METHODS

Preparation of seaweed extracts

Specimens of the red seaweed *A. spicifera* (M.Vahl) Børgesen (Rhodomelaceae, Ceramiales, Rhodophyta) were collected in May of 2013 by snorkeling to a depth of 0.5-1 m at Orla Bardot (22° 05'03" S; 41° 53'01" W) in the city of Armação de Búzios, Rio de Janeiro, Brazil. The algal material was washed with local seawater and separated from sediments, epiphytes, and other associated organisms. The air-dried algal material (204 g) was submitted to an exhaustive and sequential extraction using the following solvents in increasing polarity: dichloromethane (CH₂Cl₂ - 5X 1L), ethyl acetate (EtOAc - 5X 1L), acetone (Me₂CO - 4X 1L), and methanol (MeOH - 3X 1L) at room temperature for one week.

Cell and virus

Peripheral blood mononuclear cells (PBMCs) from healthy human donors (confidential information) were obtained through density centrifugation over Ficoll-Hypaque (Sigma) as described by Yeap et al. (2007). Cells were re-suspended in a RPMI 1640 medium

supplemented with 10% fetal bovine serum and stimulated with 5 µg/mL⁻¹ of phytohemagglutinin (PHA, Sigma) for three days and further maintained in culture medium containing 5 U/mL⁻¹ of recombinant human interleukin-2 (Sigma). The viral strain HIV Ba-L (R5-tropic) Virus type 1 was donated by the National Institutes of Health (NIH, USA) and kept in storage at -80°C.

Cytotoxicity assay

The cytotoxicity of extracts from the red seaweed *Acanthophora spicifera* was assessed by monitoring the conversion of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma-Aldrich) to formazan as previously described (Mosmann 1983) with some modification. Peripheral blood mononuclear cells were maintained in 96-well plates containing 2x10⁵ cells per well and treated with increasing concentrations of the extracts (6.75, 12.5, 25, and 50 µg/mL⁻¹ in DMSO) for one week at 37°C under a 5% CO₂ humidified atmosphere in triplicate. The DMSO concentration in the final volume of the well was less than 0.5%. After seven days of incubation, the supernatant was removed, and the MTT (20 µL of 5 mg/mL⁻¹ in medium) was added to each well. Plates were incubated for two hours at 37°C with a 5% CO₂ atmosphere. The 96 well plates were then centrifuged at 100 X g for ten minutes, the supernatant was discarded, and 100 µL of DMSO was added to each well. Finally, the optical density was measured at 545 nm on a microplate reader. The result was expressed as the 50% loss of viable cells concentration (CC₅₀). The compound concentration required to reduce the optical density of MTT in relation to not treated cells was calculated using linear regression.

Anti-HIV-1 activity in human primary cells

PBMCs were maintained in 96-well plates containing 2x10⁵ cells per well and stimulated with IL-2. PBMCs were infected with 5 ng/mL⁻¹ of the HIV-1 Ba-L strain and incubated for two hours at 37°C under a 5% CO₂ humidified atmosphere. Then, the 96 well plate was centrifuged at 100 X g for ten minutes, its supernatant was removed, new medium containing 50 µg/mL⁻¹ of extracts diluted in DMSO was added, and it was incubated at 37°C with a 5% CO₂ atmosphere for seven days. After incubation, the supernatant was collected, and the production of the p24 antigen was evaluated using the immunoassay ELISA (Zeptomatrix). The absorbance was measured in a spectrophotometer at 450 nm. Each value is expressed as mean±SEM in triplicate experiments. The average value of absorbance was used to determine the concentration of p24 in the samples treated by comparison to a standard curve (Trincherro et al., 2009).

Anti-HIV-1 RT inhibitory activity

E. coli strain BL21 (DE3) was used as a recipient for DNA transformations. Overnight, the *E. coli* cells transformed with the plasmid containing RTP66 and RTP51 HIV-1 genes were cultured in Luria-Bertani (LB) containing ampicillin (100 µg/ mL⁻¹) under shaking at 220 rpm at 37°C. These overnight cultures were used as the inoculum for one liter of LB medium containing 100 µg/mL of ampicillin. Cells were grown for six hours at 37°C with vigorous shaking and then induced with isopropyl-b-D-thiogalactopyranoside (IPTG) (1 mM) for two hours. Cells were harvested by centrifugation (5000 x g, 15 min), and bacterial lysates were prepared using a lysis buffer (50 mM Tris-HCl (pH 7.9 at 4°C), 60 mM NaCl, 1 mM EDTA, and lysozyme/DNase I treatment. Clarified lysates were used for the isolation of the p51/p66 heterodimeric RT. The active RT heterodimer was purified using the MagneHis™ Protein Purification System according to the manufacturer's instructions.

Table 1. The cytotoxicity of dichloromethane (CH₂Cl₂), ethyl acetate (AcOEt), acetone (Me₂CO) and methanol (MeOH) extracts obtained in increasing polarity from red seaweed *Acanthophora spicifera*.

Extract	CC ₅₀ (µg/mL ⁻¹)
Dichloromethane	31±7.4
Ethyl acetate	45±11
Acetone	38±3.5
Methanol	179±25

Data are expressed as mean±S.E. of three independent experiments. The CC₅₀ of each extract was calculated using regression line.

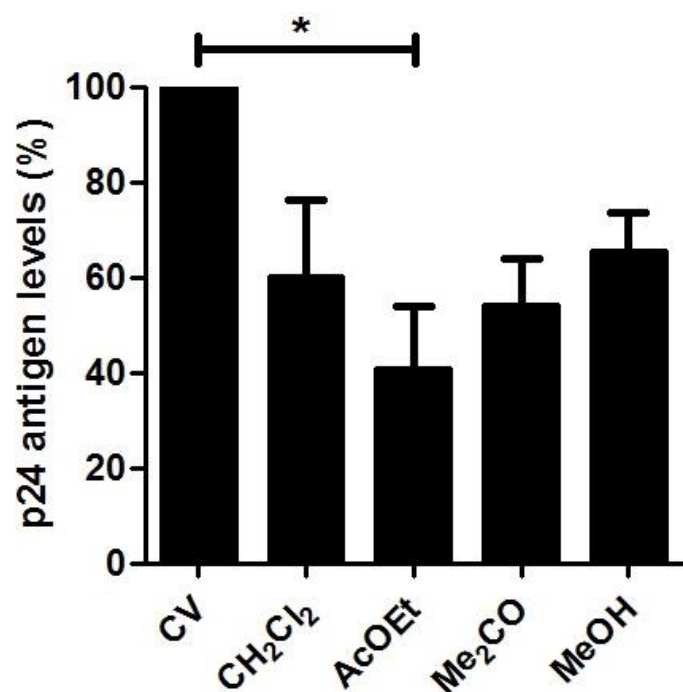


Figure 1. Histograms showing p24 levels found in the supernatant of untreated culture (Virus Control - CV) and treated with the different extracts in dichloromethane (CH₂Cl₂), ethyl acetate (AcOEt), acetone (Me₂CO) and methanol (MeOH). Each value is expressed as mean±SEM in triplicate experiments. *p<0.05, significant value as analysed by Tukey's multiple comparison test.

Table 2. Inhibitory Effects of ethyl acetate extract from Red Seaweed *Acanthophora spicifera* and Efavirenz on HIV-1 Reverse Transcriptase. Data are expressed as mean±S.E. of three independent experiments. The IC₅₀ of each extract was calculated using regression line.

Sample	IC ₅₀	Maximum inhibition
Ethyl acetate extract	33.17±4.84 µg/mL ⁻¹	79±2.1%
Efavirenz	0.006 µM	97±3.6%

The ability to inhibit the enzyme HIV-1 reverse transcriptase was evaluated using a fluorescence RT assay kit (EnzChek® Molecular Probes) according to the manufacturer's protocol. Briefly, 20 µL of

reaction mixture containing a poly(A) ribonucleotide template/oligo d(T)16 primer and dTTP was added to the wells of a microtiter plate and mixed with 5 µL of increasing concentrations of the EtOAc extract. Finally, one µL of the enzyme (15–80 ng/mL) in reaction buffer was added and incubated at 37°C for one hour. 2 µL of 200 mM EDTA was added to stop the reaction. Fluorescence intensity was measured using a microplate reader (Spectramax-M4 Molecular Devices) (ex. 480 nm, em. 520 nm) after the addition of 173 µL of fluorescent PicoGreen® reagent, which selectively binds to dsDNA or DNA-RNA heteroduplexes over single-stranded nucleic acids or free nucleotides. Efavirenz was used as a positive control. The IC₅₀ values were determined using Prism5 (GraphPad Software). All assays were performed in triplicate.

Chemical profile of extract

To analyze the chemical profile, a 5 mg aliquot of EtOAc extract was removed and analyzed by proton nuclear magnetic resonance (NMR-H). The NMR spectra were obtained on a Varian Unity Plus 300 at 300 MHz. The internal reference standard was TMS, and the samples were dissolved in CDCl₃.

RESULTS AND DISCUSSION

The CC₅₀ values of the extracts are reported in Table 1. The methanol and ethyl acetate extracts showed lower cytotoxicity in comparison with the extracts prepared in dichloromethane and acetone.

The percentages of p24 in the supernatants of each extract can be seen in Figure 1. When the culture supernatant infected with the Ba-L strain of HIV-1 was treated with 50 µg/mL⁻¹ of the extract obtained by organic solvent ethyl acetate, it was possible to observe approximately 60% reduction in the p24 levels. Treatment of infected cells with ethyl acetate extract resulted in a reduction of p24 level compared to infected untreated cells. Therefore, the extract obtained with solvent ethyl acetate was used to evaluate the ability to inhibit the activity of reverse transcriptase enzyme, an important step of HIV replication cycle.

The inhibitory activity of the EtOAc extract against HIV-1 RT is shown in Table 2. The chemical profile of ethyl acetate extract from *A. spicifera* was analyzed with NMR. The ethyl acetate extract showed signs in the chemical shift region from 7 ppm, which is characteristic of aromatic compounds (doublet in 7.53 ppm, triplet in 7.36 ppm and double doublet in 7.13 ppm).

Cytotoxicity is critical in drug development (Putnam et al., 2002). Natural seaweed products have demonstrated low levels of cytotoxicity (Karadeniz et al., 2014) – even lower than commercial drugs such as AZT (Barbosa et al., 2004). The toxicity of *A. spicifera* extracts has already been studied in mice (Naqvi et al., 1980) and Vero cell line (Duarte et al., 2004). However, the present article reports for the first time the cytotoxicity of extracts from *A. spicifera* in human cells.

When the infected culture was treated with 50 µg/mL⁻¹ of the extract prepared in ethyl acetate, there was approximately 60% decrease in p24 levels. A similar

study was conducted used partitions of CH₂Cl₂/MeOH, hexane, CH₂Cl₂, and CH₂Cl₂/EtOAc with brown seaweed *Dictyota menstrualis*. This group showed an inhibition in p24 levels of approximately 40% when tested at a concentration of 50 µg/mL (Pereira et al., 2004). Compared with the data found in this article, treatment using AcOEt extract from *A. spicifera* showed more efficient results.

The partition in ethyl acetate from brown seaweed *Ecklonia cava* had anti-HIV-1 activity, and this activity was confirmed by the presence of phenolic compounds. Other phenolic compounds such as flavonoids also showed effective anti-HIV activity (Casano et al., 2010; Wang et al., 2014). We evaluated the presence of phenolic compounds in the extracts obtained in increasing polarity from red seaweed *A. spicifera* by ¹H-NMR. Only the extract obtained with ethyl acetate showed phenolic compounds. These data corroborate the results obtained by Zeng et al. (2001), which showed the isolation of two phenolic compounds from the extract of *A. spicifera* obtained in ethyl acetate. Therefore, we believe that the anti-HIV effect of the EtOAc extract of red seaweed *A. spicifera* is due to the presence of phenolic compounds.

In a Korean study, 26 extracts from red seaweeds were tested to evaluate the inhibition of reverse transcriptase (Ahn et al., 2002). Most of the extracts were not able to inhibit more than 65% of the enzyme activity when tested at a concentration of 200 µg/mL. These data indicate that our results were interesting, since the AcOEt extract was able to inhibit 80% of enzyme activity at a lower concentration. Ahn et al. (2004) demonstrated that the inhibitory effect of brown seaweed *E. cava* was caused by the presence of phenolic compounds. In conclusion, the AcOEt extract from red seaweed *A. spicifera* presents an efficient activity against HIV-1 virus. Finally, in accordance with the observed data, the inhibitory activity of the ethyl acetate extract in HIV reverse transcriptase-1 may be due to presence of aromatic compounds.

Conflict of Interests

The authors have not declared any conflict of interest.

ACKNOWLEDGMENTS

The authors are grateful to CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support – Grant number 443930/2014-7) and for Productivity Fellowship to ICNPP (Grant number 303368/2013-6) and VLT (Grant number 304070/2014-9). ICNPP (E-26/103.024/2011) and VLT (E-26/103.176/2011) also thank the FAPERJ (Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro) for the Cientista do Nosso Fellowship and for financial support (Grant number E-26/110.205/2013). CCRN thanks

CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the PHD Fellowship.

REFERENCES

- Ahn MJ, Yoon KD, Kim CY, Min SY, Kim YU, Kim HJ, Kim JH, Shin CG, Lee CK, Kim TG, Kim SH, Huh H, Kim J (2002). Inhibition of HIV-1 reverse transcriptase and HIV-1 integrase and antiviral activity of Korean seaweed extracts. *J. Appl. Phycol.* 14(5):325-329.
- Ahn MJ, Yoon KD, Min SY, Lee JS, Kim JH, Kim TG, Kim SH, Kim NG, Huh H, Kim J (2004). Inhibition of HIV-1 reverse transcriptase and protease by phlorotannins from the brown alga *Ecklonia cava*. *Biol. Pharm. Bull.* 27(4):544-547.
- Barbosa JP, Pereira RC, Abrantes JL, Cirne dos Santos CC, Rebelo MA, Frugulhetti IC, Teixeira VL (2004). *In vitro* antiviral diterpenes from the Brazilian brown alga *Dictyota pflaffii*. *Planta. Med.* 70(9):856-860.
- Casano G, Dumètre A, Pannecouque C, Hutter S, Azas N, Robina M (2010). Anti-HIV and antiplasmodial activity of original flavonoid derivatives. *Bioorg. Med. Chem.* 18(16):6012-6023.
- Chen JY, Huang CY, Lin YS, Hwang TL, Wang WL, Chiou SF, Sheu JH (2016). Halogenated Sesquiterpenoids from the Red Alga *Laurencia tristicha* Collected in Taiwan. *J. Nat. Prod.* (Print).
- Coura CO, de Araújo IW, Vanderlei ES, Rodrigues JA, Quinderé AL, Fontes BP, de Queiroz IN, de Menezes DB, Bezerra MM, e Silva AA, Chaves HV, Jorge RJ, Evangelista JS, Benevides NM (2012). Antinociceptive and anti-inflammatory activities of sulphated polysaccharides from the red seaweed *Gracilaria cornea*. *Basic Clin Pharmacol Toxicol.* 110(4):335-341.
- Cruz-Rivera E, Villareal TA (2005). Macroalgal palatability and the flux of ciguatera toxins through marine food webs. *Harmful Algae.* 5(5):497-525.
- Duarte MER, Cauduro JP, Nosedo DG, Nosedo MD, Gonçalves AG, Pujol CA, Damonte EB, Cerezo AS (2004). The structure of the agaran sulfate from *Acanthophora spicifera* (Rhodomelaceae, Ceramiales) and its antiviral activity: Relation between structure and antiviral activity in agarans. *Carbohydr. Res.* 339(2):335-347.
- Gutiérrez-Cepeda A, Fernández JJ, Gil LV, López-Rodríguez M, Norte M, Souto ML (2011). Nonterpenoid C15 Acetogenins from *Laurencia marilzae*. *J. Nat. Prod.* 74(3):441-448.
- Hirsch MS, Günthard HF, Schapiro JM, Vézinet FB, Clotet B, Hammer SM, Johnson VA, Kuritzkes DR, Mellors JW, Pillay D, Yeni PG, Jacobsen DM, Richman DD (2008). Antiretroviral Drug Resistance Testing in Adult HIV-1 Infection: 2008 Recommendations of an International AIDS 4, Society-USA Panel. *Clin. Infect. Dis.* 47(2):266-285.
- Kaliaperumal N, Kalimuthu S, Ramalingam J, Selvaraj M (1986). Experimental field cultivation of *Acanthophora spicifera* in the nearshore area of Gulf of Mannar. *Indian J. Fish.* 33(4): 476-478.
- Karadeniz F, Kang KH, Park JW, Park SJ, Kim SK (2014). Anti-HIV-1 activity of phlorotannin derivative 8,4α-dieckol from Korean brown alga *Ecklonia cava*. *Biosci. Biotechnol. Biochem.* 78(7):1151-1158.
- Kuritzkes DR (2007). HIV resistance: frequency, testing, mechanisms. *Top HIV Med.* 15(5):150-154.
- Manhanzva MT, Mutsvangwa J, Beck IA, Frenke LM, Tshabalala M, Manasa J, Murahwa AT, Gwanzura L (2015). The burden of HIV-associated drug resistance mutations in an early infant diagnosis program: A glance through the pediatric window of Zimbabwe. *J. Infect. Dis. Ther.* 3(1):1-6.
- Marrazzo JM, del Rio C, Holtgrave DR., Cohen MS, Kalichman SC, Mayer KH, Montaner JS, Wheeler DP, Grant RM, Grinsztejn B, Kumarasamy N, Shoptaw S, Walensky RP, Dabis F, Sugarman J, Benson CA (2014). International Antiviral Society-USA Panel, 2014. HIV prevention in clinical care settings: 2014 recommendations of the International Antiviral Society-USA Panel. *JAMA.* 312(4):390-409.
- Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* 65(1):55-63.
- Naqvi-Solimabi SWA, Kamat SY, Fernandes L, Reddy CVG, Bhakuni DS, Dhawan BN (1980). Screening of Some Marine Plants from the Indian Coast for Biological Activity. *Bot Mar.* 24(1):51-55.

- Pereira HS, Leão-Ferreira LR, Moussatché N, Teixeira VL, Cavalcanti DN, Costa LJ, Diaz R, Frugulhetti ICPP (2004). Antiviral activity of diterpenes isolated from the Brazilian marine alga *Dictyota menstrualis* against human immunodeficiency virus type 1 (HIV-1). *Antiviral Res.* 64(1):69-76.
- Perrone C, Cecere E, Furnari G (2006). Growth pattern assessment in the genus *Acanthophora* (Rhodophyta, Ceramiales). *Phycologia* 45(1):37-43.
- Popplewell WL and Northcote PT (2009). Colensoide A: a new nitrogenous bromophenol from the New Zealand marine red alga *Osmundaria colensoi*. *Tetrahedron Lett.* 50(19):6814-6817.
- Silva GA, Domingos TFS, Fonseca RR, Sanchez EF, Teixeira VL, Fuly AL (2015). The red seaweed *Plocamium brasiliense* shows anti-snake venom toxic effects. *J. Venom Anim. Toxins Incl. Trop. Dis.* 21(2):1-9.
- Trincherro J, Ponce NM, Cordoba OL, Flores ML, Pampuro S, Stortz CA, Salomon H, Turk G (2009). Antiretroviral activity of fucoidan extracted from the brown seaweed *Adenocystis utricularis*. *Phytother. Res.* 23(5):707-712.
- Vo TS, Kim SK (2010). Potential anti-HIV agents from marine resources: An overview. *Mar. Drugs* 8(12):2871-2892.
- Wang WX, Qian JY, Wang XJ, Jiang AP, Jia AQ (2014). Anti-HIV-1 activities of extracts and phenolics from *Smilax china* L. *Pak. J. Pharm. Sci.* 27(1):147-151.
- Yeap SK, Alitheen NB, Ali AM, Omar AR, Raha AR, Suraini AA, Muhajir AH (2007). Effect of *Rhaphidophora korthalsii* methanol extract on human peripheral blood mononuclear cell (PBMC) proliferation and cytolytic activity toward HepG2. *J. Ethnopharmacol.* 114(3): 406-411.