Journal of Medicinal Plant Research

Volume 8 Number 34, 10 September, 2014
ISSN 2009-9723

Academic Journals

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Journal of Medicinal Plants Research

Table of Contents: Volume 8 Number 34, 10 September, 2014

ARTICLES

Research Articles

academicJournals

Vol. 8(34), pp. 1065-1073, 10 September, 2014
DOI: 10.5897/JMPR2014.5481
Article Number: C5A2D7E47404
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plant Research

Full Length Research Paper

Medicinal plants from Saudi Arabia and Indonesia: *In vitro* cytotoxicity evaluation on Vero and HEp-2 cells

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Received 2 June, 2014; Accepted 1 September, 2014

Many types of naturally growing plants are used traditionally for the treatment of different types of cancers and infectious diseases. In this report, the cytotoxic activity of 30 medicinal plants, commonly used in folk medicine in Saudi Arabia and Indonesia, was evaluated *in vitro* using Vero and HEp-2 cell lines. Plants were randomly chosen and harvested from different districts of both countries based on ethnobotanical information and subsequently extracted by methanol. Serial two-fold dilutions of each extract, starting from the concentration of 1000 µg/ml, were incubated with Vero and HEp-2 cells for 72 h. The cytotoxic effect of different extracts was *in vitro* characterized by identification of cellular alterations microscopically and cellular viability colormetrically. The plant extracts were classified according to the minimal toxic concentration and 50% cytotoxicity concentration indexes into three groups: highly cytotoxic (8 to 31 µg/ml), moderately cyototoxic (32 to 499 µg/ml) and low cyototoxic (500 to 1000 µg/ml). The results showed that the extracts of *Juniperus phoenicea* and *Calotropis procera* were highly cytotoxic on both cell lines to the minimal concentration of 1 µg/ml and may be well-considered as potential candidates for anticancer research. Two more extracts (*Datura inoxia* and *Citrullus colocynthis*) produced significant cytotoxicity to the minimum concentration of 16 µg/ml, with selective powerful activity of *C. colocynthis* on HEp-2 cells. The other extracts showed lower degrees of cytotoxicity and may be utilized for testing as antiviral agents using cell culture models.

Key words: *Calotropis procera*, cell titer blue assay, cytotoxic activity, *Juniperus phoenicea*, medicinal plants, methanolic extracts.

INTRODUCTION

Medicinal plants have emerged as a promising source of novel therapeutic agents due to their higher structural diversity and potency as compared to standard synthetic chemistry. In general, medicinal plants are cheap, easy to obtain and widely used in many countries such as China, India, Indonesia and a number of Middle East countries to treat various infectious diseases (Guo et al., 2006; Mukhtar et al., 2008). The World Health Organization has reported that about 80% of the world population depends on traditional medicine for their primary health care

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(Farnsworth et al., 1985; Gurib-Fakim, 2006). According to the estimate of Robbers et al. (1996), over 25% of modern medicines that are commonly used worldwide contain compounds extracted from medicinal plants. Medicinal plants provide the source of extremely useful active compounds that are difficult to produce commercially by synthetic methods. They also supply basic compounds that can be modified slightly to render them more active, effective and less toxic (Robbers et al., 1996).

Previous studies have shown that active compounds extracted from medicinal plants have considerable inhibitory effect on cancer cells (Moogkarndia et al., 2004; Takara et al., 2005; Shoeb, 2006; Nawab et al., 2011), bacteria (Tsibangu et al., 2002; Rangasamya et al., 2007; Akroum et al., 2009; Marzouk et al., 2012) and many viruses such as herpes simplex, human immunodeficiency, hepatitis B and influenza viruses (Rajbhandari et al., 2001; Mukhtar et al., 2008; Devi and Manoharan, 2009), beside their potential as antioxidants (Gupta et al., 2006; Krishnaiah et al., 2011). The use of medicinal plant extracts for cancer prevention and treatment was extensively studied (Mukhtar et al., 2008; Mehta et al., 2010). Extracts of medicinal plants are believed to contain a wide spectrum of polyphenolic, flavonoids, alkaloids, terpenoids and saponin compounds, which might have therapeutic properties and hinder cancer formation (Dai and Mumper, 2010). The isolation of the alkaloid active compounds vinblastine and vincristine from *Charanthus roseus*, has introduced a new era for the use of plant materials as anticancer agents (Shoeb, 2006).

In a recent record, about 60% of the currently used anticancer drugs have been isolated from natural products; mostly of plant origin (Nawab et al., 2011). Saudi Arabia and Indonesia are considered among the richest regions with plant diversity and harbors very important genetic resources of medicinal plants in the Arabian Peninsula and Southern Asia, respectively. More than 2,250 species of medicinal plants were estimated to be used for medical purposes in local communities of Saudi Arabia (Rahman et al., 2004). In Indonesia, the use of medicinal plants in traditional medicine was practiced long time ago using what is called 'Jamu', which is a combination of various medicinal plant preparations. A minimum of 151 medicinal plant species that belong to 57 families have been utilized as substantial materials of Jamu (Riswan and Roemantyo, 2002). Nevertheless, only few of the medicinal plants in both countries have been evaluated for their potential as anticancer agents (Amin and Mousa, 2007). Therefore, it is very interesting to explore more about the great probability of medicinal plants from Saudi Arabia and Indonesia for the treatment of cancer diseases. In this present report, based on ethnobotanical approach, we have evaluated *in vitro* the cytotoxic activity of 30 medicinal plants, commonly used by traditional healers in both countries, using Vero and HEp-2 cell lines.

MATERIALS AND METHODS

Plants selection and collection

An assortment of 30 medicinal plants was chosen for the cytotoxicity testing based on: first, previous literature reviews and second, ethnobotanical information. The selected plants that belong to different family groups were collected from different districts of the Kingdom of Saudi Arabia (KSA) and Indonesia (IND) (Table 1).

Preparation of plant extracts

Plant samples were dried at room temperature and finely ground with a hammer mill. For *Aloe vera,* the leaves were cut and the gel was squeezed out in a sterile container. Each 20 g of powdered plant material or gel was extracted by maceration overnight with 200 ml methanol at room temperature. After filtration, methanol was evaporated under reduced pressure till complete dryness and the crude extract was dissolved in dimethyl sulphoxide (DMSO) to a final concentration of 50 mg/ml. All crude extracts (stocks) were kept at -20°C for cytotoxicity testing procedures.

Cell culture

African green monkey kidney cells (Vero) and human larynx cancer cells (HEp-2) were kindly provided by Virology Research Group (VRG), College of Science, King Saud University, Saudi Arabia. Cells were cultured in Dulbecco's modified eagle's medium (D-MEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY), 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml) (Sigma, St. Louis, MO). Cells treated with medicinal plant extracts were kept in maintenance medium containing 1% FBS, L-glutamine and antibiotics. All cells were incubated at 37°C with 5% CO_2.

In vitro evaluation of the cytotoxicity of plant extracts

Microscopic examination for morphological alterations

Monolayer cultures of Vero and HEp-2 cells (80 to 90% confluence) were prepared in 96 well plates. After removal of culture medium, cells were washed twice with phosphate buffered saline. Two-fold serial dilutions of the crude plant extracts were prepared in maintenance medium starting from the concentration of 1000 µg/ml, and added to cells in triplicates. Wells that received methanol 70% were served as positive controls and those that received maintenance media only were served as negative controls. All cultures were kept at 37°C in CO_2 incubator for 72 h with daily observation for morphological changes. Cellular alterations were recognized in the form of cell rounding, granulation, vacuolation, degeneration and lysis, as well as detachment of the monolayer. The minimal toxic concentration (MTC) was identified as the least concentration of the plant extract preparation that induce toxic effect(s) on culture cells as detected microscopically after 72 h of incubation.

Cell viability assay

In this assay, the number of viable cells was determined colorimetrically in 96-well plates. In independent set of experiment, following the incubation of confluent monolayer cultures of cells with two-fold dilution series of plant extracts for 72 h, 20 µl of CellTiter-Blue (CTB) reagent (Promega, Madison, WI) were added to each well. After 4 h of incubation at 37°C, the optical density (OD) was measured in all plate wells using ELx880 microplate reader (BioTek, Winooski, VT) with wavelength 570 nm. The cell viability was measured in each well using

Table 1. List of medicinal plants utilized in the current study.

S/No	Scientific name	Family	Common name	Part of plant used	Selected traditional uses	Collection site
1	*Chamomilla recutita*	Asteraceae	Chamomile	Flower	Fever, inflammation, insomnia, ulcer, wound	Riyadh, KSA
2	*Dodonaea viscosa*	Sapindaceae	Hop bush	Leaves	Fever, gout, rheumatism, hemorrhoid, malaria	Tabuk, KSA
3	*Punica granatum*	Lytharaceae	Pomegranate	Peel	Diarrhea, cough, diuretic, vomiting, jaundice	Riyadh, KSA
4	*Psidium guajava*	Myrtaceae	Guava	Leaves	Diarrhea, dysentery, stomach, fever	Riyadh, KSA
5	*Hibiscus sabdariffa*	Malvaceae	Roselle	Flower	Diuretic, sedative, stomach, fever, liver	Riyadh, KSA
6	*Mentha piperita*	Lamiaceae	Peppermint	Leaves	Bronchitis, analgesic, colds, sore throat	Madinah, KSA
7	*Mentha longifolia*	Lamiaceae	Mint	Leaves	Bronchitis, carminative, analgesic, colds	Madinah, KSA
8	*Capparis spinosa*	Capparaceae	Caper bush	Leaves	Diuretic, astringent, tonic, stomach, headache	Tabuk, KSA
9	*Capparis cartilaginea*	Capparaceae	Caper	Leaves	Rheumatism, diabetes, antihelmintic	Tabuk, KSA
10	*Calotropis procera*	Asclepiadaceae	Sodom apple	Leaves	Tumors, liver, skin diseases, diabetes	Riyadh, KSA
11	*Juniperus phoenicea*	Cuppriscaea	Juniper	Leaves	Diarrhea, rheumatic, bronchitis, arthritis	Tabuk, KSA
12	*Citrullus colocynthis*	Cucurbitaceae	Bitter apple	Fruit	Jaundice, tumors, constipation, fever, scabie	Riyadh, KSA
13	*Foeniculum vulgare*	Apiaceae	Fennel	Leaves	Carminative, digestive, gastrointestinal	Riyadh, KSA
14	*Ziziphus spina-christi*	Rhamnaceae	Christ's Thorn Jujube	Leaves	Fever, pain, dandruff, inflammatory, wounds	Riyadh, KSA
15	*Senna indica*	Caesalpinaceae	Cassia	Leaves	Laxative, thypoid, cholera, jaundice, gout	Riyadh, KSA
16	*Salvia verbenaca*	Lamiaceae	Wild Sage	Leaves	Wounds, astringent, diuretic, antiseptic	Riyadh, KSA
17	*Lawsonia inermis*	Lythraceae	Henna	Powder	Diuretic, constipating, inflammations, cough	Riyadh, KSA
18	*Aloe vera*	Xanthorroeaceae	Aloe	Gel	Burning, skin irritations, tonic, shampoos	Riyadh, KSA
19	*Datura inoxia*	Solanaceae	Thorn-apple	Leaves	Anodyne, pain relief, wounds, hemorrhoids	Serpong, IND
20	*Curcuma xanthorrhiza*	Zingiberaceae	Temulawak	Rhizome	Skin inflammations, indigestion, constipation	Serpong, IND
21	*Curcuma zedoaria*	Zingiberaceae	Zedoary	Rhizome	Vomiting, stomach ulcers, menstrual	Serpong, IND
22	*Curcuma longa*	Zingiberaceae	Turmeric	Rhizome	Diarrhea, stomach ulcers, skin diseases	Serpong, IND
23	*Zingiber officinale*	Zingiberaceae	Ginger	Rhizome	Digestive disorder, arthritis, vomiting	Serpong, IND
24	*Andrographis paniculata*	Acanthaceae	Andrographis	Leaves	Leprosy, scabies, anti-inflammatory, diuretic	Serpong, IND
25	*Anredera cordifolia*	Bacellaceae	Madeira Vine	Leaves	Skin, gout, hypertension, inflammation	Serpong, IND
26	*Typhonium flagelliforme*	Araceae	Rodent Taro	Root	Expectorant for cough, pulmonary ailments	Serpong, IND
27	*Phyllanthus niruri*	Phyllanthaceae	Meniran	All parts	Diabetes, fever, cough, diarrhea, dysentery	Serpong, IND
28	*Allium sativum*	Alliaceae	Garlic	Bulb	Cough, colds, dysentery, hypertension	Serpong, IND
29	*Coriandrum sativum*	Apiaceae	Coriander	Fruit	Diabetes, ulcers, diuretic, liver, skin	Serpong, IND
30	*Momordica charanthia*	Cucurbitaceae	Bitter Melon	Fruit	Stomach, diabetes, anthelmintic, jaundice	Serpong, IND

KSA = Kingdom of Saudi Arabia; IND = Indonesia.

well using the following formula:

$$\text{Cell viability} = \frac{\text{OD (assay well)} - \text{OD (positive control)}}{\text{OD (cell control)} - \text{OD (positive control)}} \times 100$$

Table 2. Cytotoxic activity of plant extracts against Vero and Hep-2 Cells by microscopical examination.

Scientific name	Vero (Concentration, µg/ml)								HEp-2 (Concentration, µg/ml)							
	1000	500	250	125	64	32	16	8	1000	500	250	125	64	32	16	8
Chamomilla recutita	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
Dodonaea viscosa	+	+	+	-	-	-	-	-	+	+	+	+	-	-	-	-
Punica granatum	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-
Psidium guajava	+	+	+	-	-	-	-	-	+	+	-	-	-	-	-	-
Hibiscus sabdariffa	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-
Mentha piperita	+	+	-	-	-	-	-	-	+	+	+	-	-	-	-	-
Mentha longifolia	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Capparis spinosa	+	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Capparis cartilaginea	+	+	+	+	+	-	-	-	+	+	+	+	+	+	-	-
Calotropis procera	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Juniperus phoenicea	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrullus colocynthis	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	-
Foeniculum vulgare	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-
Zizipus spina-christia	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-
Senna indica	+	+	+	+	-	-	-	-	+	+	+	-	-	-	-	-
Salvia verbenaca	+	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-
Lawsonia inermis	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-
Aloe vera	+	+	+	-	-	-	-	-	+	+	+	+	-	-	-	-
Datura inoxia	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-
Curcuma xanthorrhiza	+	+	+	-	-	-	-	-	+	+	+	+	+	-	-	-
Curcuma zedoaria	+	+	+	+	+	-	-	-	+	+	+	+	-	-	-	-
Curcuma longa	+	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-
Zingiber officinale	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-
Andrographis paniculata	+	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-
Anredera cordifolia	+	+	+	+	+	-	-	-	+	+	+	+	+	-	-	-
Typhonium flagelliforme	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-
Phyllanthus niruri	+	+	+	+	-	-	-	-	+	+	+	+	+	-	-	-
Allium sativum	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-
Coriandrum sativum	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-
Momordica charanthia	+	+	+	-	-	-	-	-	+	+	+	+	-	-	-	-

+indicates obvious morphological alterations; -indicates no cellular changes.

The 50% cytotoxicity concentration (CC_{50}) was calculated as the concentration of the plant extract that induced reduction in cell viability to 50%.

RESULTS

In the present study, the cytotoxic activity of thirty (30) medicinal plants collected from Saudi Arabia and Indonesia and also representing twenty two (22) plant families (Table 1) was evaluated *in vitro* according to their effect on cell morphology (microscopic examination) and the metabolic reduction of CTB reagent (colorimetric assay) Vero and HEp-2 cells. The cytotoxic effect of plant extracts was classified into three groups; highly cytotoxic (8 to 31 µg/ml), moderate cyototoxic (32 to 499 µg/ml) and low cyototoxic (500 to 1000 µg/ml). This classification was consistent with both MTC and CC_{50} indexes for the majority of plant extracts. Vero cells, 3 plant extracts (10%) were highly cytotoxic, 22 (73.3%) were moderately cytotoxic, and 5 (16.7%) were low cytotoxic as indicated by MTC index. Whereas using CC_{50} index, 2 plant extracts (6.7%) were highly cytotoxic, 19 (63.3%) were moderately cytotoxic, and 9 (30%) were low cytotoxic. On the other hand, the plant extracts were distinguished in HEp-2 cells as: 4 (13.3%) highly cytotoxic, 21 (70%) moderately cytotoxic and 5 (16.7%) low cytotoxic using MTC index; and 2 (6.7%) highly cytotoxic, 23 (76.7%) moderate cytotoxic and 5 (16.7%) low cytotoxic using CC_{50} index (Figure 1).

Out of the eight medicinal plant species used in the study, one viz, *Juniperus phoenicea* belongs to Gymnospermae while the rest belong to Angiospermae. The extracts of *J. phoenicea* and *Calotropis procera* exhibited a substantial degree of cytotoxicity Vero and HEp-2 cells. Both extracts were capable to induce distinct morphological alterations in cell culture, as evaluated by microscopical examination (Table 2 and Figure 2), and effective inhibition of cell viability, as measured by CTB assay (Table 3 and Figure 4), down to the concentration of 8 µg/ml. Further evaluation of lower concentrations of the two extracts outlined that the cytotoxic activity can extend down to the concentration of 1 µg/ml (data not shown). Two other extracts; *Datura inoxia* and *Citrullus colocynthis*, were included in the highly cytotoxic group albeit their effect is lower than *J. phoenicea* and *C. procera* and is not well-consistent in microscopical examination and CTB assay (Tables 2 and 3 and Figures 3 and 4). The extract of *Datura inoxia* was

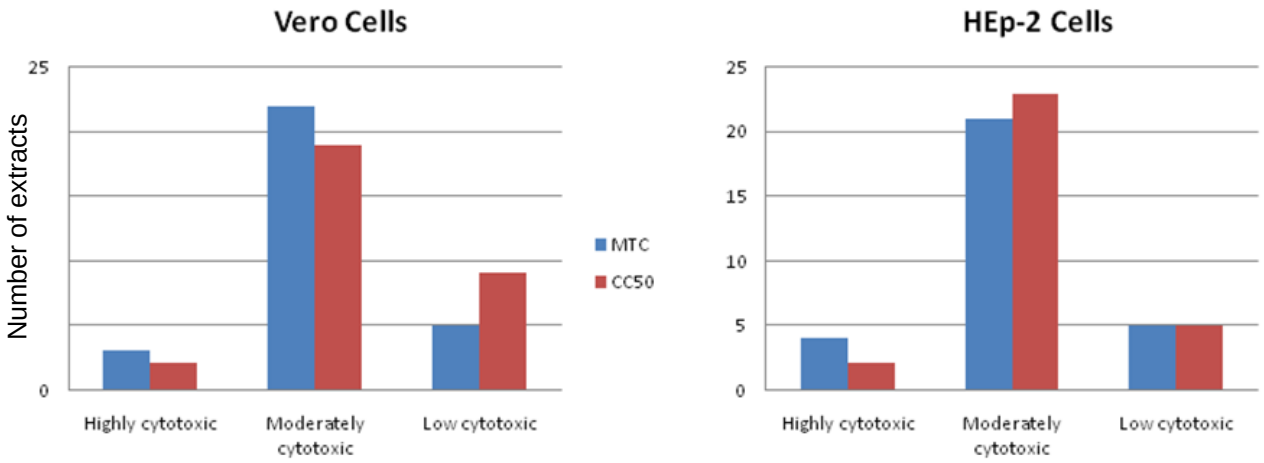

Figure 1. Cytotoxicity of the plant extracts Vero and Hep-2 cells. The different medicinal plants are classified into three categories according to their effect on culture cells as indicated by MTC and CC_{50} indexes; highly cytotoxic (8 to 32 µg/ml), moderately cyototoxic (32 to 499 µg/ml) and low cyototoxic (500 to 1000 µg/ml).

Table 3. The limit of cytotoxic activity of plant extracts Vero and HEp-2 cells as indicated by MTC and CC_{50}.

Scientific name	Vero		HEp-2	
	MTC*	CC_{50}*	MTC	CC_{50}
Chamomilla recutita	1000	>1000	1000	>1000
Dodonaea viscosa	250	382.77	125	266.85
Punica granatum	250	667.46	250	369.26
Psidium guajava	250	402.86	500	402.68
Hibiscus sabdariffa	250	500.89	250	445.65
Mentha piperita	500	724.95	250	337.91
Mentha longifolia	500	>1000	500	>1000
Capparis spinosa	1000	>1000	500	>1000
Capparis cartilaginea	64	182.15	32	72.16
Calotropis procera	8	5.96	8	4.9
Juniperus phoenicea	8	6.63	8	29.79
Citrullus colocynthis	64	109.39	16	37.78
Foeniculum vulgare	125	589.58	125	546.51
Zizipus spina-christia	250	452.66	250	403.2
Senna indica	125	204.69	250	452.49
Salvia verbenaca	64	73.65	64	114.36
Lawsonia inermis	250	308.11	250	334.28
Aloe vera	250	598.7	125	250.95
Datura inoxia	16	54.98	16	110.22
Curcuma xanthorrhiza	250	374.84	64	92.61
Curcuma zedoaria	64	75.33	125	282.69
Curcuma longa	32	45.88	32	50.13
Zingiber officinale	125	218.31	125	264.96
Andrographis paniculata	125	569.18	64	125.57
Anredera cordifolia	64	77.99	64	112.62
Typhonium flagelliforme	250	729.23	250	274.65
Phyllanthus niruri	125	426.17	64	120.73
Allium sativum	250	324.86	250	442.66
Coriandrum sativum	500	585.24	500	813.63
Momordica charanthia	250	667.39	125	297.27

*Concentrations are expressed as µg/ml. MTC: Minimum toxic concentration. CC_{50}: 50% cytotoxic concentration.

effective on both cell lines down to the concentration of 16 µg/ml microscopically and 54.98, 110.22 µg/ml as determined by CTB assay on Vero and HEp-2 cells, respectively. In contrary, *C. colocynthis* extract was much potent on HEp-2 cells (MTC: 16 µg/ml; CC_{50}: 37.78) than on Vero cells (MTC: 64 and 109.39 µg/ml).

DISCUSSION

Medicinal plants remain a major source for the development of new anticancer drugs (Fouchea et al., 2008). Recent scientific research has shown that many plants used in traditional medicine are potentially toxic, allergic, mutagenic, and/or carcinogenic (Ahmad et al., 2006; Akintonwa et al., 2009). Therefore, *in vitro* cytotoxic evaluation programs are important to obtain effective anticancer agents that have certain desirable properties such as: little or no toxic effects on normal cells; high efficacy on multiple sites; capability of oral consumption; known mechanism of action; low cost, and acceptance by human population (Aziz et al., 2003). Cytotoxicity testing of medicinal plants is not only important to evaluate and validate the safety of medicinal plants for traditional use, but also provides guidance in the search for new active compounds.

In the present study, the cytotoxicity of 30 plant extracts was evaluated Vero and HEp-2 cells indicating variable degrees of cellular degeneration. The extracts of *J. phoenicea* and *C. procera* showed the most powerful cytotoxicity in both cell lines (Figure 2). Comparable results were previously obtained by testing the cytotoxic activity of the essential oils of *J. phoenicea* leaves and berries on a variety of cancer cell lines derived from brain (U251), lung (H460), liver (HepG2), breast (MCF7) and cervical (Hela) tissues (El-Sawi et al., 2007). Ethanolic root extracts of wild *C. procera* also showed, marked *in*

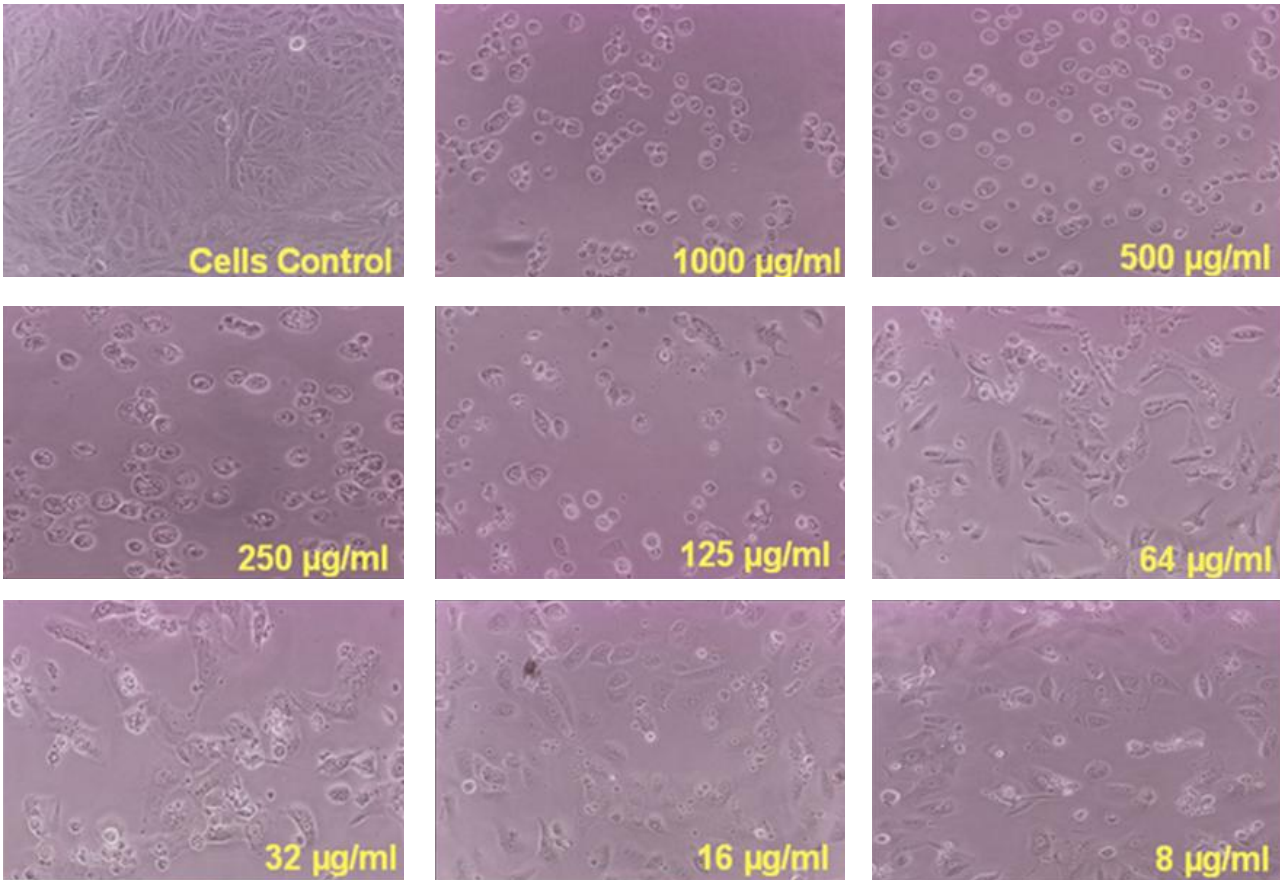

Figure 2. A tracking record of the morphological changes Vero cells after treatment with *J. phoenicea* extract (representation for the effect of highly cytotoxic group of plant extracts on both cell lines).Various concentrations of the extract, ranging from 1000 to 8 µg/ml, were incubated with Vero cells for 72 h. Cellular alterations were recorded and photographs were captured using inverted microscope at 100 magnification (Eclipse TS100, Nikon, Japan).

vitro cytotoxicity against cancer cells of oral (KB) and nervous (SNB-78) origin at three different concentrations; 10, 30 and 100 µg/ml (Bhagat et al., 2010). Moreover, the results obtained by Murti et al. (2012) demonstrated that leaves of *C. procera* extracted by N-butanol possess strong cytotoxic activity against HEp-2 cells with a mean CC_{50} value of 3.7 µg /ml.

In this regard, it is worthy to mention that the mean CC_{50} value of both extracts in our analysis were lower than 30 µg/ml (5.95 and 6.63 Vero cells, and 4.9 and 29.79 on HEp-2 cells for *J. phoenicea* and *C. procera*, respectively). According to the standards of U.S. National Cancer Institute (NCI), the CC_{50} value of a good anticancer candidate should be lower than 30 µg/ml to avoid unspecific effects (Suffness and Pezzuto, 1990). Therefore, these two extracts may be considered as promising candidates for further evaluation against different kinds of tumors both *in vitro* and *in vivo.* Although the CC_{50} values of *Datura inoxia* and *C. colocynthis* extracts are exceeding 30, their potent cytotoxic effect may be considered for further evaluation using other cell types. The same theory could be applied on certain extracts of the moderately cytotoxic group, which are capable to induce cytotoxicity down to a concentration of 32 µg/ml (microscopically) and 72 µg/ml (using CTB assay) like *Curcuma longa, Capparis cartilaginea, Salvia vertbenaca* and *Anredera cordifolia*.

Phytochemical analysis of *J. phoenicea* and *Calotropis procera* extracts indicated the existence of different potential compounds such as flavonoids, alkaloids, tannins, saponins and phenolics (Mossa et al., 1991; Qnais et al., 2005; Hayouni et al., 2007; Moronkola et al., 2011; Medini et al., 2013). Flavonoid and phenolic compounds are important groups of medicinal plant metabolites which have various biological activities in the control of cancer and other human diseases (Ghasemzadeh and Ghasemzadeh, 2011). Flavonoids are widely distributed natural products of medicinal plants with more than 2000 different active compounds of wide-spectrum potential (example flavonos, flavanones, flavonols, anthocyanidins and isoflanos) (Robbers et al., 1996). Whereas, certain phenolic compounds, like pyrogallol and catechol showed powerful antimicrobial activity in different study sets (Mason and Wasserman, 1987). Further experiments are

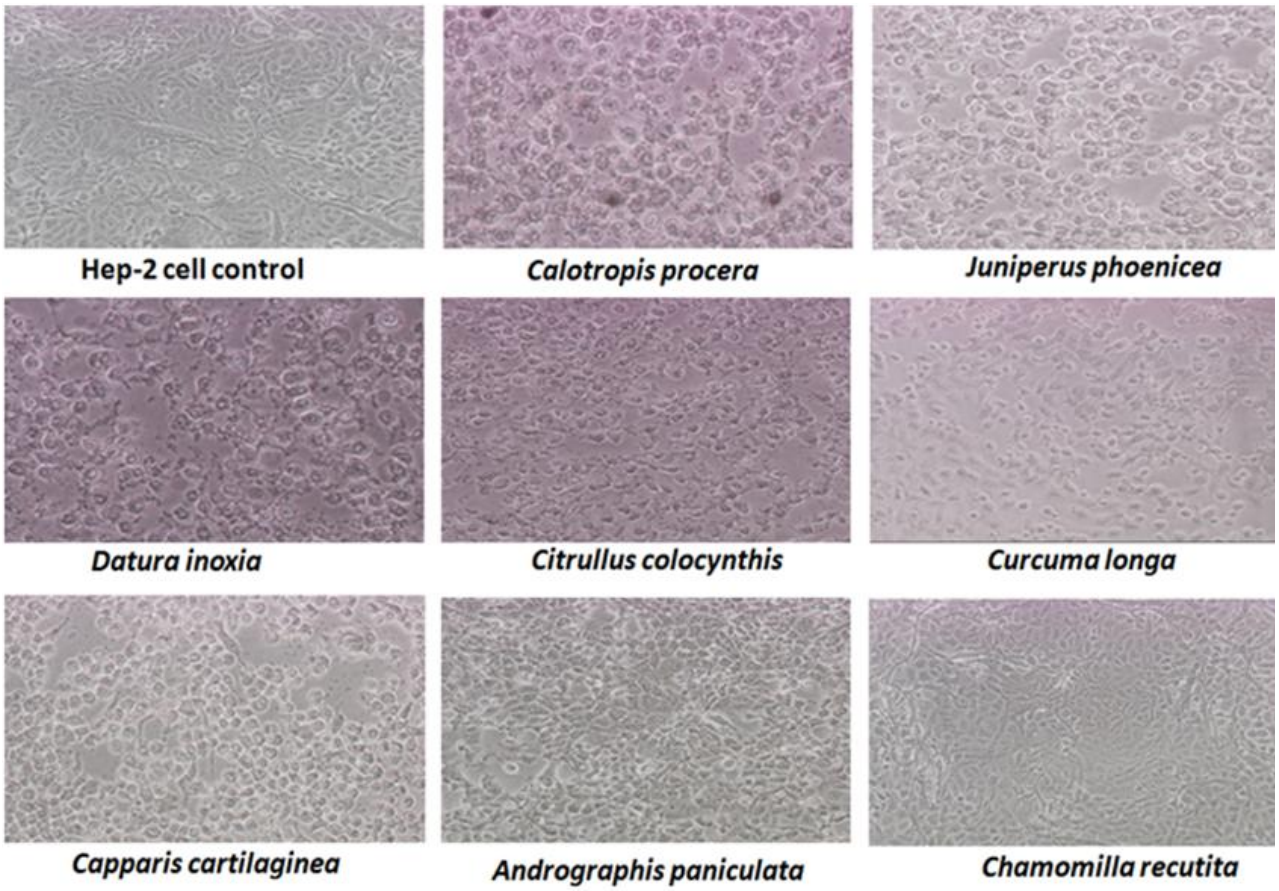

Figure 3. Morphological changes of Hep-2 cells after treatment with different plant extracts. Cells were incubated with the extracts of *C. procera, J. phoenicea, D. inoxia, C. colocynthis, C. longa, C. cartilaginea, A. paniculata* and *C. recutita* at a concentration of 64 µg/ml for 72 h. Cellular alterations were recorded in comparison to untreated cells and photographs were captured using inverted microscope at 100× magnification (Eclipse TS100, Nikon, Japan).

required to isolate pure active compounds from these plant extracts of high cytotoxicity and to determine their anticancer activities using different cancer cell lines and animal models.

A recent study demonstrated that *Datura inoxia* leaves, which are extracted using organic solvents like petroleum ether, are five times more toxic than aqueous extracts in mice (Kutaifa et al., 2012). The superior toxicity of organic extracts was proposed as regarded to the presence of active compounds such as flavonoids and essential oils that are soluble in organic solvents but not in water. However, other investigators claimed that aqueous extracts can induce apoptosis of cancer cells through activation of caspase-3 and -9, and suppression of vascular endothelial growth factor (VEGF) and tumor necrosis factor-alpha (TNF-α) (Pandey et al., 2011). The potent cytotoxic effect of methanolic extracts of *Datura inoxia* in cell culture in the current study further potentiate the concept of using organic solvents for such purpose. However, more comprehensive studies are necessary to justify the role of different active compounds of both types of extracts in combating cancer cells. On the other hand, the selective cytotoxicity of *C. colocynthis* on HEp-2 cells is an observation that was recorded before using the ethanolic extract at a concentration of 100 µg/ml (Afshari et al., 2005). This may suggest a degree of affinity toward epithelial cell carcinomas. Inclusion of several cell lines of different sources will justify this speculation empirically. The majority of the plant extracts tested in this study (18 out of 30) showed MTC and CC_{50} values that did extend below the concentration of 125 µg/ml (Tables 2 and 3). This level of cytotoxicity leaves considerable range of non-cytotoxic concentrations that enable testing of such extracts on both cell lines for potential antiviral activity. Vijayan et al. (2004) demonstrated that the replication of herpes simplex virus on Vero cells was completely inhibited by the extracts of *Hyparicum mysorense and Hypericum hookerianum* at concentrations of 100 and 50 µg/ml, respectively. Similarly, the extracts of *Hydroclathrus clathrus* and *Lobophora variegata* held up the infectivity of respiratory syncytial virus on HEp-2 cells at concentrations of 100 and 25 µg/ml, respectively

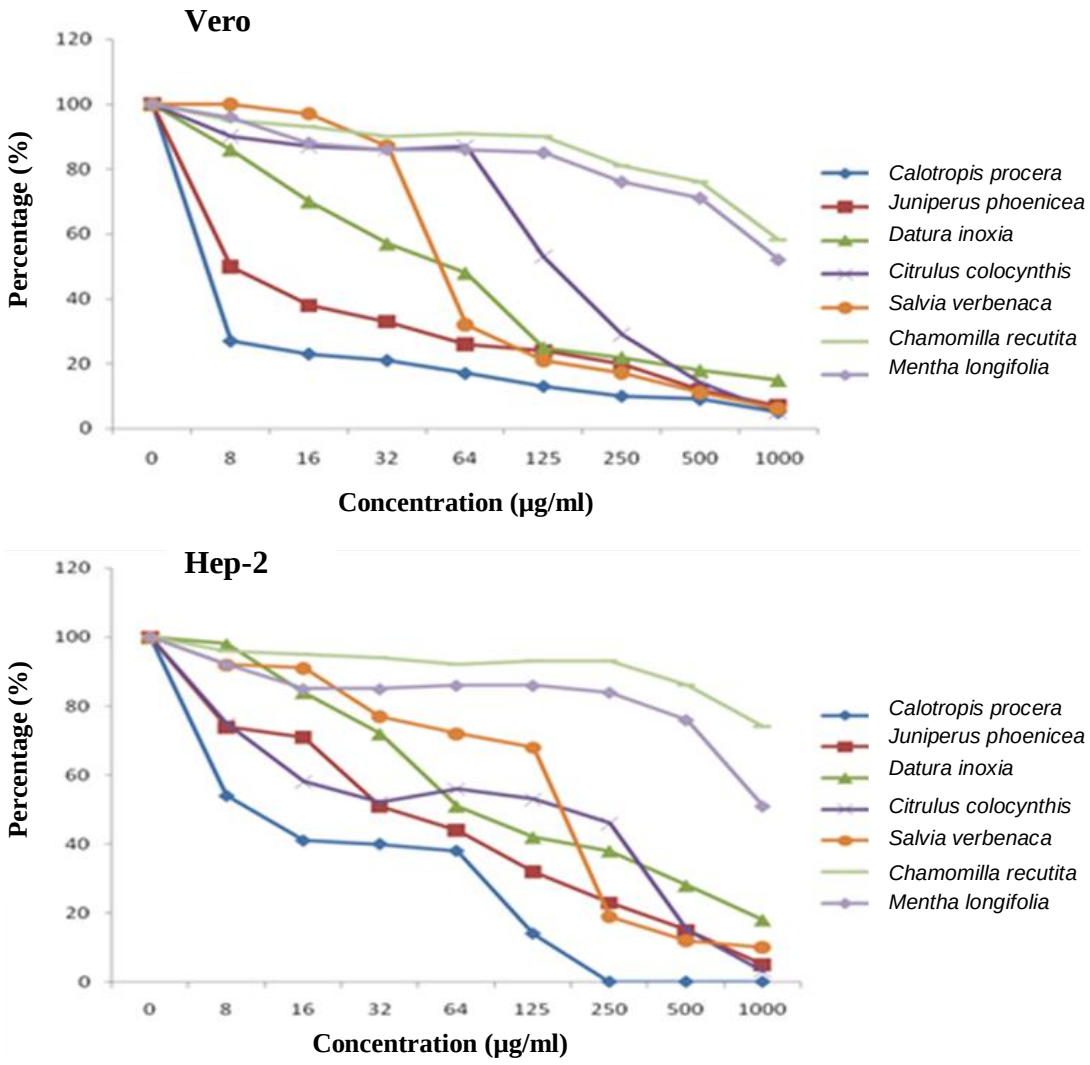

Figure 4. Cell viability plot of representative plant extracts as measured by CTB assay. Vero and Hep-2 cells were incubated for 72 h with extracts of *Calotropis procera, Juniperus phoenicea, Datura inoxia, Citrullus colocynthis, Salvia berbenaca, Chamomilla recutia* and *Menta longifolia.* Cell viability was determined for each extract at various concentrations ranging from 1000 to 8 µg/ml using cell titer blue assay.

(Wang et al., 2008).

Conclusion

The cytotoxic activity of 30 medicinal plants harvested from Saudi Arabia and Indonesia was evaluated Vero and HEp-2 cells. The extracts of *J. phoenicea* and *C. procera* leaves were highly cytotoxic on both cell lines even with minute concentrations, while the extracts of *D. inoxia* and *C. colocynthis* were cytotoxic down to the concentration of 16 µg/ml, with selective effect of the latter on HEp-2 cells. The majority of extracts showed cytotoxic activity of limited extent, which allows the use of both cell types as models for evaluating the potential effect of these plant extracts against viral infections.

ACKNOWLEDGMENT

The authors extend their appreciation to the Dean of Scientific Research at King Saud University for funding the work through the research group project no. RGP-VPP-180.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Vol. 8(34), pp. 1074-1080, 10 September, 2014
DOI: 10.5897/JMPR2014.5466
Article Number: 38B004147405
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plant Research

Full Length Research Paper

Development of a topical gel containing dried extract of *Ipomoea pes-caprae brasiliensis* (L.) R. Br. (Convolvulaceae)

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Received 3 May, 2014; Accepted 25 August, 2014

Gel formulations containing *I. pes-caprae* spray dried extract (SDE) at 0.11 and 0.18%, were developed with three types of polymers Ce = Cellosize® QP 100, Ca = Carbopol® ultrez, and Ar = Aristoflex® AVC. The pH, sensory characteristics, weight loss and the viscosity profile of gels were analyzed at times (t) t_0, t_{90} and t_{180} (24 h, 90 and 180 days after their production, respectively), in ambient temperature (AT = 25 ± 2 °C), in the fridge (F = 5 ± 2 °C) and in the oven (OV = 40 ± 2 °C). Isoquercitrin assay were analyzed by High-performance liquid chromatography (HPLC), after 24h and 360 days (in AT and in the F). None of these formulations nor the spray dried extract (SDE) showed any level of cutaneous irritation in agarose overlay test. The greatest polymers were Ca and Ar in relation to their sensory characteristics and viscosity.

Key words: *Ipomoea pes-caprae*, gel, spray dried extract, stability, cytotoxicity.

INTRODUCTION

Ipomoea pes-caprae brasiliensis (L.) R. Br. (Convolvulaceae), popularly known in Brazil as “Salsa da Praia”, is a typical sandbank plant, which naturally occurs in tropical and sub-tropical coastal regions worldwide (Christman, 2000; Castellani and Santos, 2006). It has been used in folk medicine to treat wounds caused by jellyfish venom (Pongprayoon et al., 1991a, 1991b). Antinociceptive and anti-inflammatory properties (De Souza et al., 2000), among a wide range of pharmacological activities like antioxidant, antispasmodic, antihistaminic, insulogenic and hypoglycemic activities have been reported in an extended review (Manigaunha et al., 2010). From aerial parts, both methanol and hydro-alcoholic extracts have already revealed potent and significant anti-nociceptive and anti-inflammatory properties, in the abdominal contortions models and the pleurisy models, respectively (De Souza et al., 2000; Vieira et al., 2013). Among the vehicles for topical use, the choice for

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the choice for gels is mostly due to the absence of grease substances, the sensory of refreshment, as well as the tendency to spread easily by friction (Chorilli et al., 2006). Some of the most common products used to producing gels are the polymers as hydroxyethyl cellulose, acrylic acid polymers and ammonium acryloyl dimethyltaurate/ carboxyethyl acrylate co-polymers. The cellulose-based derivatives as hydroxyethyl cellulose, like Cellosize®, are an important class of polymers with many industrial applications.

It is an important non-ionic biodegradable polysaccharide that has interesting hydrophilic, rheological, as thickener or binder, and antibacterial properties, producing transparent films (Martinez-Richa, 2012). Carbopols® are insoluble acrylic acid polymers, which became stiff gels upon neutralization in aqueous medium. Carbopol® Ultrez presents better dispersion properties and a potential wide range of applicability in the pharmaceutical field, with high viscosity at low concentration, compatibility, bioadhesion and good user acceptance. Aristoflex® AVC correspond to a co-polymer of ammonium acryloyl dimethyltaurate/ carboxyethyl acrylate, forming transparent gels in aqueous systems, with good spreading and stability over pH from 4 to 9, promoting a soft sensation in the skin (Tamburic & Craig, 1995; Contreras et al., 2001). Taking into account the traditional use and the pharmacological results which have been related for ethanol extract of *I. pes-caprae* aerial parts, the present work was designed to develop topical gel formulations containing *I. pes-caprae* spray dried extract (SDE), which could be safe and proper to be used for the treatment of algesic and inflammatory skin diseases.

MATERIALS AND METHODS

Isoquercitrin (> 95% of purity by HPLC) was purchased from Sigma Aldrich (St. Louis, Missouri, USA). Colloidal silicon dioxide and Aristoflex® AVC were purchased from Pharma Special (São Paulo, Brazil). Cellosize® QP 100, imidazolinidilureia and Nipagin® were purchased from Via Farma (Ipiranga-SP, Brazil). Carbopol® Ultrez and propylene glycol were purchased from Galena (Campinas-SP, Brazil), 2,2'-azino-di-(3-ethyl benzthiazoline-6-sulphonic acid (ABTS) from Fluka (São Paulo, Brazil), sodium dodecyl sulphate (SDS) from Biomatec (Rio de Janeiro, Brazil), minimum essential medium (MEM), phosphate buffered saline (PBS) from Gibco (São Paulo, Brazil) and $NaBH_4$ from Nuclear (São Paulo, Brazil). The ethanol was analytical grade, purchased from Dinâmica (Diadema, São Paulo Brazil). All other solvents were analytical grade. Methanol and acetonitrile (J.T.Baker®, Phillipsburg, USA) were HPLC grade. High purity water (18 MΩ) was provided by an Easy pure water system (Waltham, Massachusetts, USA) fed with reverse osmosis water.

Plant material

The botanical material was collected from Esplanada beach (Jaguaruna, Santa Catarina, Brazil) in February 2007. A voucher was deposited at the Barbosa Rodrigues Herbarium (Itajaí, Santa Catarina, Brazil) under number V.C. Filho 009. The leaves, stems and other aerial parts (flower buds, seeds and flowers) were manually separated, cleaned and dried in an air oven at 35 ºC until moisture stabilization. After drying, plant material was ground in a hammer mill (outlet sieve = 3 mm) and the average size of the particles was determined by sieving (Allen et al., 2007).

Extractive solution

At first, the extractive solution (ES) was obtained by maceration over 7-day period, with 12.5% (*w/v*) plant ratio in alcohol 70 °GL, as previously related by Vieira et al. (2013). Before spray drying, ES was pre-concentrated under vacuum until 40% reduction of its original volume. The percentage of dried extractives in ES was determined gravimetrically at 105 °C, according to the method described in the Brazilian Pharmacopoea (Brasil, 2010). Each 5.0 g of samples were analyzed in quadruplicate.

Spray dried extract

The spray dried extract (SDE) of *I. pes-caprae* (SDE) were obtained in a Mini-Spray Dryer (Büchi 290, Flawil, Switzerland), with two components nozzle and co-current flow, nozzle aperture of 0.7mm, spraying pressure of 5 bar, flow of 4 mL min^{-1}, inlet temperature of 170°C, aspiration of 90% and air flow rate of 400 NL h^{-1}, by co-drying with 20% (*w*/*w*) of Aerosil® (in relation to dry residue of previous concentrated extractive solution). The residual humidity was determined in an infrared weight scale (MettlerToledo, LJ16, Switzerland) and expressed as the average percentage of three determinations. The morphology of the dried product was determined using scanning electron microscopy (SEM) performed on a PhilipsXL30 microscope, and representative samples of selected drying processes were put over a metallic support covered with colloidal gold under vacuum.

Gel formulations

To develop gel formulations, three types of polymers were used (Ce = Cellosize® QP 100, Ca = Carbopol® Ultrez and Ar = Aristoflex® AVC) in two concentrations each (Ce = 1 and 2%; Ca = 1 and 2%; Ar = 2.5 and 5%) and two SDE concentrations (0.11 and 0.18%). Each formulation was composed by SDE, propilenoglycol (0.2%), Nipagin® (0.2%), imidazolidinyl urea (0.3%) and purified water. The pH of all formulations was adjusted to 5.5 to 6.5 by adding citric acid or sodium hydroxide solution, respectively. The gels were stored in aluminum tubes, and placed in different conditions as described in the accelerated stability study.

Accelerated stability study

The pH, sensory characteristics, weight loss, viscosity profile of gels were analyzed at different times (t) (t_0, t_{90} and t_{180} corresponding to 24h, 90 and 180 days respectively, after their production), at ambient temperature (AT = 25 ± 2 °C), in a fridge (F = 5 ± 2 °C) and in an oven (OV = 40 ± 2 °C). Chromatographic profile and isoquercitrin contents, in the highest polymer and SDE concentrations, were analyzed by HPLC at time 0 (t_0) and after 360 days (t_{360}) and at AT and F. Gel formulations which demonstrated the best performance in the stability studies, as well the SDE itself, were evaluated by *in vitro* cytotoxicity through the agarose overlay

method.

Viscosity analysis

Gel samples (1 g) were analyzed in a rotational viscometer, cone-plate sensor Haake VT 550 PK 1 1°, 25 °C, coupled to a circulating water bath (25 ± 1 °C). Flow curves were analyzed to characterize the viscosity (mPa.s), flow index and thixotropy of the formulations.

HPLC analysis

A previous validated method (Vieira et al., 2013) for assay of isoquercitrin in SDE sample, that showed linearity in the range of 10 to 110µg mL^{-1} (r^2 > 0.99), repeatability and intermediate precision with relative standard deviation (RSD%) < 15%, and 97.57 to 107% of isoquercitrin recovery was used in this study in order to preview the chemical stability of gel formulations, after extraction steps. A Shimadzu LC-10AD HPLC system (Shimadzu, Tokyo, Japan) equipped with a binary pump and a SPD-M10A photo diode array detector, a CTO-10A column oven, and an automatic injection system was used. The mobile phase consisted of acidified water pH 3.2 as solvent A and acetonitrile: methanol (50: 50, *v/v*) as solvent B, gradient system of 75:25 for 30min, to 60:40 on 20min, 35 °C and flow rate of 0.8mL min^{-1}, through a C_{18} 5 µm 100 Å (250 x 4.6 mm) column (Phenomenex®, Torrance, USA), with detection at 254nm and injection of 20µL. All gel samples (1.0g) were extracted with the aid of centrifugation with methanol (5.0mL) and sodium chloride salt (0.1g). The supernatant was dried under hot air and the dry residue was dissolved with methanol (2.0mL) and filtered through 0.2µm membranes (Cromafil® PET-20 µm /15 mm) (Macherey-Nagel Inc. USA) before injection. The isoquercitrin assay in the samples was determined by external standardization, using an analytical curve built with the authentic sample of the marker.

The recovery of the extraction method was carried out by spiking the placebo with 200 µL of standard solution of isoquercitrin at 120 µg mL^{-1}, to obtain a final concentration, in the sample solution of 12µg mL^{-1}, within the linearity of the method.

***In vitro* citotoxicity**

The agarose overlay method was used as an *in vitro* test to evaluate the cutaneous irritation of developed gel formulations. For that, L929 cells were obtained from the Rio de Janeiro cell bank. These were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% PSN (peniciline, steptomicine and neomicine) antibiotic and 0.5% antifungal amphotericin B, at 37 °C with 5% CO_2 and 95% air. Cells were plated at 1000 cells / well in DMEM, remaining incubated for 24h at 37 °C with 5% CO_2 and 95% air. After this time, the medium was replaced with DMEM serum medium absent FCS containing 0.01% neutral red as vital dye. Cells remained in the oven for 3h at 37 °C and 5% CO_2 for appearance of red staining. The culture medium was removed and replaced again by mixing 1:1.2 agarose medium and MEM medium (mixture overlay) maintained under heating at 40 °C to prevent solidification methods. The mixture was placed in overlay plates (6 sterile wells containing 1 x 10^6 cells) and these were maintained in a 37 °C oven and 5% CO_2 for 24h. After that, the samples were applied in triplicate on disks with 0.5cm diameter and positive controls (latex and SDS) and negative control (solution 73 saline) were incubated for 24h at 37 °C and 5% CO_2. Besides SDE and developed gel formulations, propylene glycol, and other herbal gel acquired from the market were also evaluated. The degree of irritation was evaluated by the zone of lysis (no incorporation of vital dye), with the aid of microscopic visualization and calipers. The evaluation criteria were followed according to United States Pharmacopoeia (2006).

RESULTS AND DISCUSSION

The batch of herb raw material used for this study had 38.46% of stems and 61.54% of leaves, with an average particle size of 0.537mm. The 12.5 % (*w/v*) hydroalcoholic solution presented a dry residue of 2.35± 0.11 % (*w/w*). The extract SDE was obtained with an arithmetic mean yield of 61 ± 2.83%, taking into account the total solids into the feed solution of spray drying. The moisture content was 5.92 ± 0.063%, fulfilling the acceptance criteria for hygroscopic powders such herbal derivatives (List and Schmidt, 1989). SDE was characterized as a fine powder, with a yellow-brown color. The SEM (Figure 1) showed agglomerated and spherical particles with smooth surfaces with a size range between 2 and 10 µm.

In this study, all the formulations maintained pH values from 5.5 to 6.5, which could be appropriate for topical use. However, the presence of SDE caused a strongly darkening in all of the samples stored in OV (t_{180}). This effect was mostly evident in the Ce gels, including AT (t_{90}) and F (t90), which not only suffered a darkening but also a heterogeneous aspect in AT (t_{90}) and OV (t_{180}). The weight loss ranged from 0.4% (Ca 1A/AT t_{90}) and 18.13% (Ar 2.5C/OV t_{180}). OV was the condition that most influenced weight loss of the formulations. Comparatively, there was a tendency to greater dehydration of the formulations containing Aristoflex® AVC gel, and most formulations containing Cellosize® QP 100 showed less tendency to dehydration, regardless of storage conditions. Probably, this behavior is associated with the water-retaining properties of this polymer, since all formulations contain propylene glycol as humectant. The viscosity was stable up to 90 days at AT, nevertheless tending to be reduced in F and OV (t_{90}), and higher at AT and OV (t_{180}) (Figure 2). All the gel formulations showed pseudoplastic flow and a characteristic thixotropic profile until the end of the study. All formulations increased the viscosity, depending on the polymer concentration, and decreased from 8 to 15% with the presence of SDE.

The viscosity had not suffered great modifications over 90 days at AT, but in the F and OV the viscosity of all formulations reduced around 24%. In the end of 180 days, this effect was reproduced in the formulations at F, with a viscosity reduction of 16%. However, after 180 days, the viscosity increased for all the formulations with Carbopol® 2% and SDE in AT, in the F and OV. As this effect was not direct correlated with the weight loss of formulations, some other kind of polymeric interactions

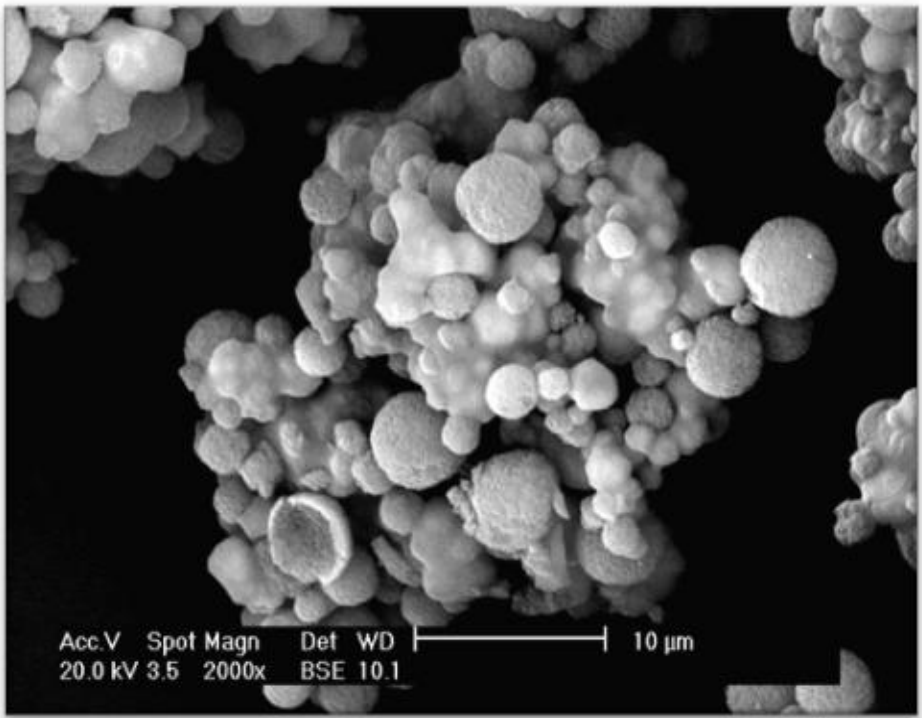

Figure 1. Microscopic image of the SDE samples of *I. pes-caprae* obtained by scanning electron microscopy.

might be related. There are formulations in which a decrease of viscosity was observed during the storage, probably due to the entrapment of bubbles air into the gel. Formulations of Cellosize® base plus SDE showed a heterogeneous aspect after 90 and 180 days in the OV and F conditions. This phenomenon did not allow the viscosity measurements and might be related to interactions between the hydroxyethyl cellulose and SDE. The extraction and quantification methods for HPLC analysis of isoquercitrin (Iso) in the SDE of *I. pes-caprae* (Vieira et al., 2013) were adapted for the Ca and Ar gels in AT and Ce in F (Figure 3). The Iso content was reduced to 6.5% in the Ca in AT (t_{360}). In the Ar gels, stored in F (t_{360}), new peaks were observed in the chromatogram (Figure 3), suggesting degradation products. Only the Ce gel stored in the F (t_{360}) maintained 75% of Iso at the end of this study (Table 1), but with a significant decrease in viscosity. The results of the content of isoquercitrin are related to the marker recovery difficulties. The recovery was 98.2% isoquercitrin (RSD% = 0.99%) for the gel with Carbopol® Ultrez, 73.54% (RSD% = 3.4%) for Aristoflex® gel and only 44.6% (RSD% > 15%) for the gel with Cellozise® 100 QP, due to the nature of the gel suggesting that studies using other clean-up techniques must be employed in this formulation. Only after 1 year, in the fridge, it was possible to measure the concentration of Iso from the gel with Cellozise® QP 100, but at the expense of the loss of viscosity.

The cytotoxicity test diffusion by agarose overlay is indicated to emulsions and gels in aqueous continuous phase, related to potential irritation risk (ANVISA, 2004). Samples of propylene glycol, *I. pes-caprae* dry extract and gels containing or not SDE were evaluated. All samples showed no irritation halo, indicating that there is no degree of mucosa/skin irritation. The positive controls showed halos 4, classified as severe reactivity. The phytopharmaceutical gel, analyzed as the reference gel with anti-inflammatory properties (negative control), showed halos 3, classified as “moderate reactivity”. Therefore, gels containing Ar and Ca with SDE were approved in the issue of security by cytotoxicity test. The results in this work were prompt to show a greater feasibility for the production of gels containing 0.18% dry extract of *I. pes-caprae* from Ca and Ar bases, taking into account their visual characteristics and viscosity. In addition, none of them as well as the SDE, showed any level of cutaneous irritation. Further studies of stability and degradation should be done with herbal raw material and intermediate products such as dry extracts and concentrated extractive solution, in order to elucidate their chemical stability as well as other possible vehicles or formulations should be evaluated for stability improvement.

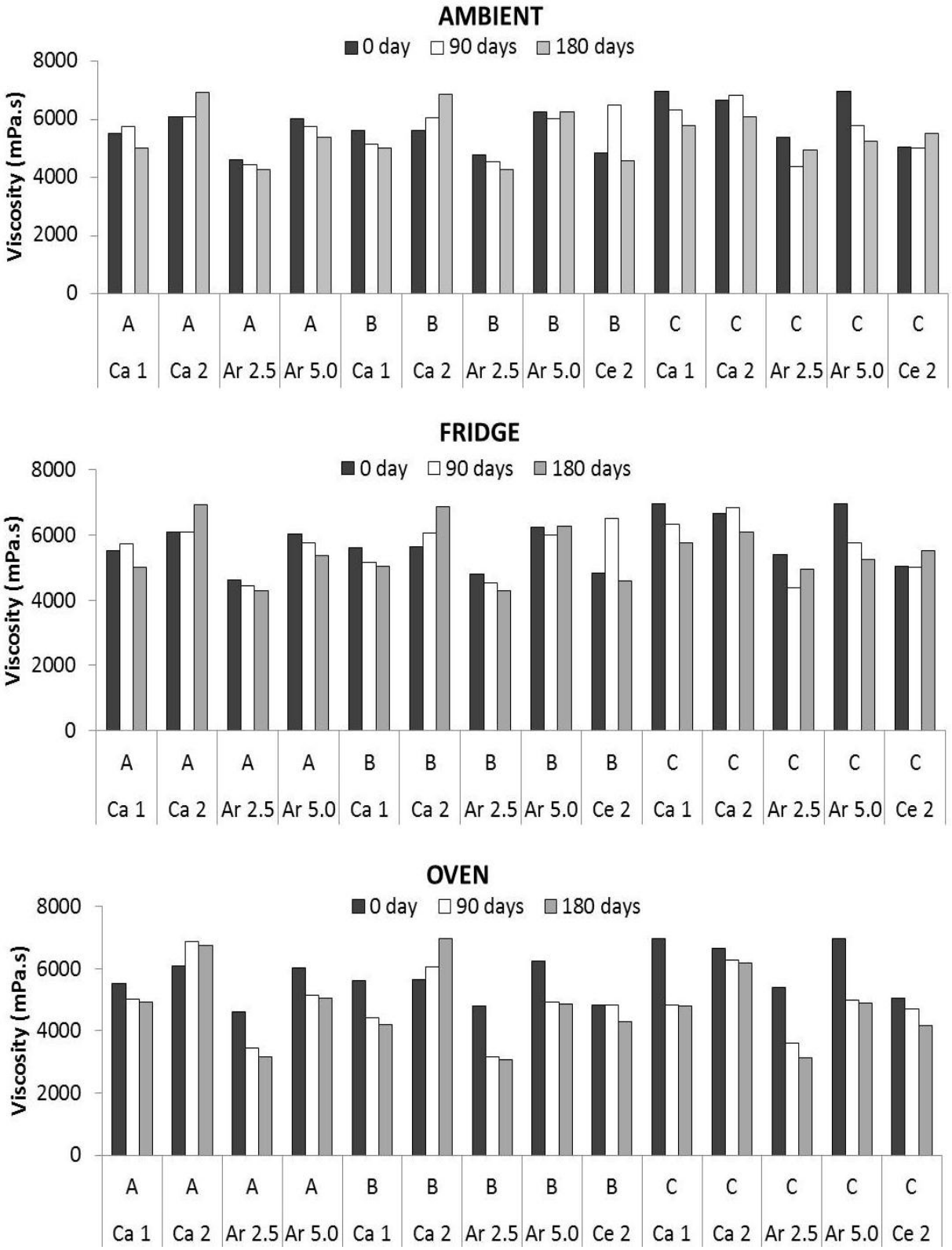

Figure 2. Viscosity measurements of gel formulations in ambient temperature (AT), in the fridge (F) and oven (OV). Ar = Aristoflex® AVC; Ca = Carbopol® ultrez and Ce = Celulosize® QP100. A = 0.11% of SDE; B = 0.18% of SDE; C = 0.0 of SDE. 1, 2, 2.5 and 5 are the polymer concentrations (%).

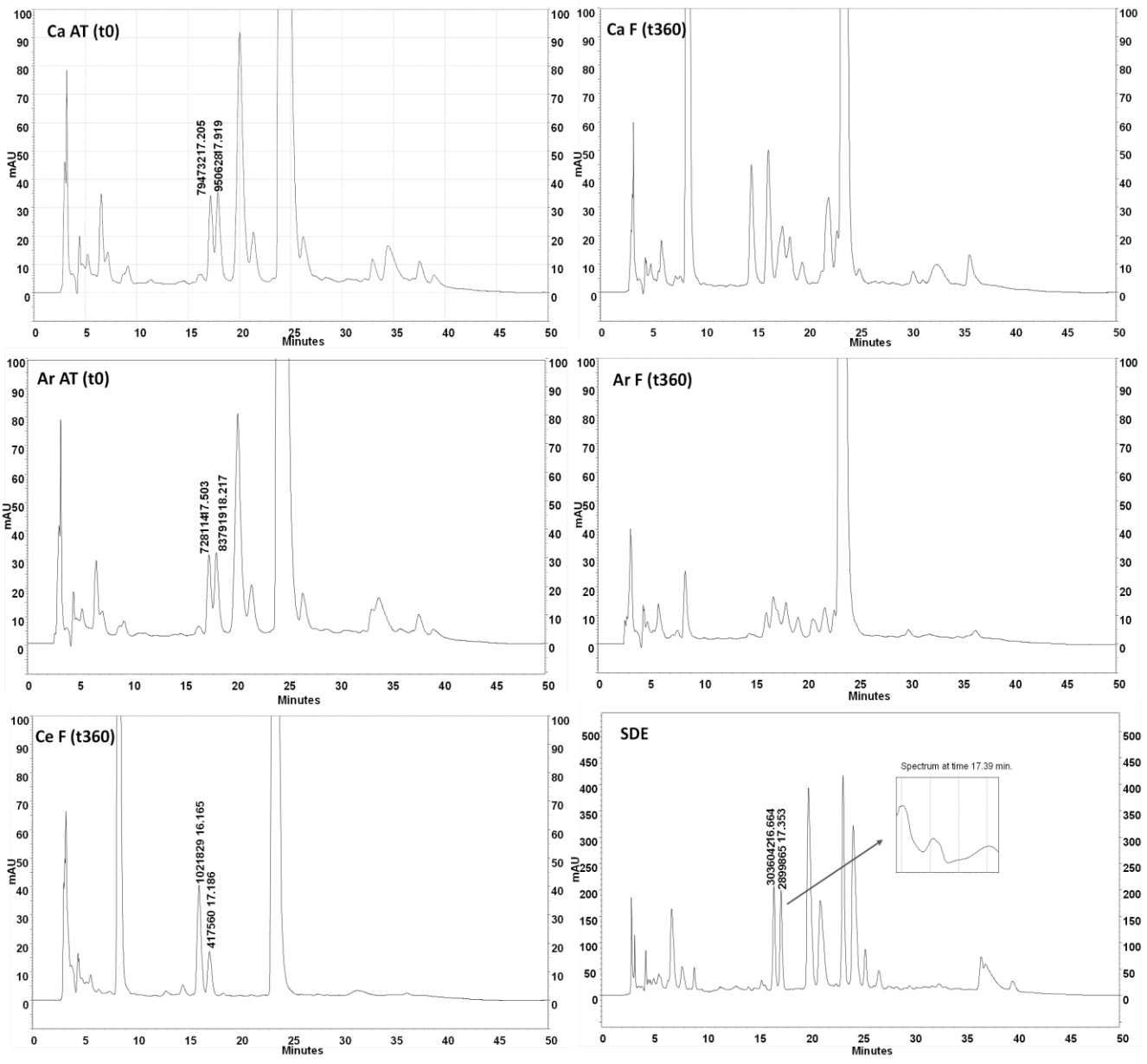

Figure 3. Chromatographic profile of SDE (solution at 10 mg/mLpeak at 18 min = isoquercitrin), Ca (2%), Ar (5%) and Ce (2%) gels containing 0.18% of SDE, monitored at 254 nm.

conclusion

In conclusion, this work provides useful information about physical and chemical stability of gels containing a dry extract of an herbal derivative. Despite that some of the commercial topical formulas are available in the market, with cosmetic or therapeutic purposes; this work demonstrated how difficult it is to obtain a gel formulation with an acceptable quality. The problems were mostly related with the viscosity, as well the chemical marker content over the time.

Conflict of interests

The author(s) have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors are grateful to FAPESC (Pronex Edital) for

Table 1. Isoquercitrin concentration (Iso) in the gels containing 0.18% of spray dried extract of *I. pes-caprae* over stability study by HPLC.

Gel	Ambient temperature		Fridge
	Iso (μg/mL) at T_0	Iso (μg/mL) at T_{360}	Iso (μg/mL) at T_{360}
Aristoflex® AVC	94.67 (7.41)	6.42 (3.27)	ND
Carbopol® ultrez	99.33 (8.04)	6.17 (8.13)	ND
Celulosize® QP100	13.58 (26.79)	13.67 (4.86)	73.33 (4.27)

ND = not detectable; T_0 = time after 24 h; T_{360} = storage for 360 days; In parenthesis, relative standard deviation (RSD%)

financial support

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academicJournals

Vol. 8(34), pp. 1081-1085, 10 September, 2014
DOI: 10.5897/JMPR2013.5296
Article Number: 72F27EE47406
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plant Research

Full Length Research Paper

Fatty chemical composition and antioxidant activities of coconut oils (*Cocos nucifera* L)

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Received 4 November 2013; Accepted 27 August, 2014

Fixed oils from two cultivars (green and yellow) of solid albumen in the coconut palm (*Cocos nucifera*), were obtained by a solvent extraction using hexane in three different maturation stages, such as: unripe; ripe and dried. The aforesaid oils were saponified and methylated. Fatty acids and methyl esters were analyzed by the gas chromatography, mass spectrometry (CG-MS). The oils from unripe (green and yellow) coconuts, as major constituents, were identified with hydrocarbons, thioesters and carboxylic acids, as well as the major compounds being 69 and 65%, respectively. The oils for the ripe coconut, having major compounds, corresponded to 74 and 70% for the green and yellow variation, respectively. In these said experiments, a common set of fatty acids were detected. Therefore, for the dried coconut, the main constituents corresponded to 99.98 and 98.11% (green and yellow) variations. The results of the free radical scavenging effects in the fixed oil from the coconuts of the both cultivars, showed a concentration-dependent activity with IC_{50} varying from $5.2x10^{-6}$ to $1.1x10^{-4}$.

Key words: Coconut oil, fatty acid, chemical composition, antioxidant activity.

INTRODUCTION

The coconut tree is one of the most important palm trees cultivated in the tropics (Laureles et al., 2002); it belongs to the *Arecaceae* and it is characterized by three drupaceous ovaries confined in the fruit with a rigid endocarp of three germinal pores, as well as the fruit being usually a seed (Ejedegba et al., 2007). These species, in turn, are composed of some varieties, whereby the most important are the “typica” (Giant variety) and “nana” (dwarf) varieties). The latter, is composed by yellow and green varieties, Malaysia´s red and Camerron´s red cultivars (Aragão et al., 1999). The *natura* for daily delicious and nutritional beverage. This palm tree plays an important role as a subsistence crop thought its commercial oil (Aragão et al., 2002). The dried coconut is only a stage where the shell undergoes dehydration and simultaneously, the liquid albumen decreases and the water

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the water juice dries (Cascudo, 1983). The coconut oil called “oleo-de-coco” is divided into two categories: virgin and refined. The vigin oil type is obtained starting from fresh fruits (ripe coconut). On the green cultivar is very common in Brasil and it is used *in* flip side, the refined oil is obtained typically from the dried coconut, named “Copra” (Kabara, 1990). According to the ethnobotanical knowledge, the coconut for oil extraction, is chosen based on their weight and the amount of water it has. In order to see if it is in good condition, it is good enough to hit it with a coin in its shell. If it is cool, the sound is loud; if the sound is hollow, it indicates that the fruit is rotten and has oxidized. Daily published reports showed that a considerable percentage of the coconut oil, is constitute carbon chain C6-C12 (Lópes-Villalobos et al., 2001).

The main constituents of the coconut fixed oil are the triacylglycerols and the carbon chain do not exhibit an unsaturation degree. In this particular case, the major portion is due to the lauric acid and the myristic acids (dodecanoic acid), followed by a small amount of the linoleic acid (Pham et al., 1998; Nevin and Rajamohan, 2006; Laureles et al., 2002). Concerning the biological benefits, the comparison between virgin and Copra oils showed that the former presents higher benefits than the Copra oil, such as a reduction of the levels in the total cholesterol, phospholipids and LDL-c, as well as an increase in the tissular levels and the series HDL-c (Guo et al., 2006), antithrombotic effect, fibrinogen, factor V, 6-ketoPGF1E, Prothrombin time (Nishi et al., 2005), besides showing an antioxidant activity. Despite the antioxidant activity in the virgin oil promoting a reduction in the lipid peroxidation *in vitro,* as well as *in vivo* (Nevin and Rajamohan, 2006), and a reduction of the abdominal fat in the men, besides the loss of corporal weight and the reduction of the total fat mass (Guo et al., 2006; Nishi et al., 2005; Nevin and Rajamohan, 2008; Stonje et al., 2003; Stonge and Bosarge, 2008; Stonge and Jones, 2002; Hargrave et al., 2005).

The oxidative process is associated in several diseases, such as the cardiac and the Alzheimer in our continuous research for natural antioxidants. The activities of oils from the coconut were evaluated. The coconut juice was previously reported to be a source of antioxidants. The present work has the purpose to determine and compare oil compositions in the coconut from two varieties, each one in three different maturation stage: unripe, ripe and dried, besides the analysis of antioxidant activities in these oils.

MATERIALS AND METHODS

Plant material

The fruits samples in the *Cocos nucifera,* were harvested in the city of Icarai (Geographical Coordinates: 3° 41' 0" South, 38° 40' 0" West), State of Ceará, Brazil in February, 2011. The plant identification was done by Edson P. Nunes and the voucher specimens # 30848 and 30849 have been deposited in the Herbario Prisco Bezerra’s, Biology Department of the Federal University of Ceara in Brazil.

Assays

Extraction of the Fixed Oil from the Solid Coconut Albumen

The unripe, ripe and dried of the green and yellow coconuts (albumen, Figure 1), were cut in small pieces of about 1cm. The cut pieces (200g) were placed in an Erlenmeyer 500mL and extracted with the hexane (200mL) for 48 hours in room temperature. The solid material was then filtrated in vacuum and solvent, as well as evaporated under a reduced pressure. The duly obtained weights (g) and yields (%) respectively were: unripe 1G (2.0/1.0), 1Y (2.2/0.4), ripe 2G (3.9/2.0), 2Y (2.7/1.4) and dried 3G (7.0/2.2), 3Y (5.0/1.6). The residue were named 1G, 2G, 3G, 1Y, 2Y and 3Y oils for the green/yellow coconut in the stages as unripe, ripe and dried, respectively.

Saponification/ methylation of the coconut oils

Each of the oil samples, 2.0g (1G, 2G, 3G, 1Y, 2Y and 3Y), were dissolved in 15.0mL of MeOH (11.85g/0.37mol) with a stoichiometric amount of the NaOH (0.37mol/14.8g) and left to react in room temperature for two hours. The solvent residue evaporated until dryness. The yielded saponified compounds duly obtained are summarized as follows: unripe 1G (2.4 g), ripe 2G (4.3g), dried 3G (5.2g), unripe 1Y (2.1g), ripe 2Y (3.8g) and dried 3Y (5.4g). The methylation from each oil was carried out using the MeOH (10mL) in acidic medium (2mL of HCl) heating during 1.5h. The reacted products were submitted to a chromatography column in silica gel, using hexane and chloroform (9:1) as eluents. The products were analyzed in the GC/MS. (Table 1).

Antioxidant activity

This spectrophotometer assay used the stable radical DPPH, as a reagent (Burits and Bucar, 2000; Hegazi and El Hady, 2002). These aforementioned experiments were carried out with oil samples, before the derivatization. The experiments consisted in preparing seven concentrations, each fixed with oil and mixed with the same volume of 60μM DPPH solution (e.g., 500μL). This particular analysis was measured in the spectrophotometer at 520nm and the inhibition of the free radical DPPH (in percent, I%) was calculated using the formula below:

$$I\% = [1 - \frac{A_{sample}}{A_{blank}}] \cdot 100$$

Where the A_{blank} is the absorbance in the control reaction (containing all the reagents, except the oil sample), while the A_{sample} is the absorbance of the test sample. The IC_{50} value was calculated from the plot of the inhibition percentage against the sample concentration. These said tests were carried out in triplicate. Six samples (oil unripe green, oil unripe yellow, oil ripe green, oil ripe yellow, oil dried green and oil dried yellow), as well as the DPPH were dissolved in ethanol. The Trolox and the BHT were used as a positive control and the results are presented on Table 2.

Statistical analysis

The results are expressed as mean ± S.E.M. However, the one-way analysis of the variance (ANOVA) was used. Whereas, in the

Figure 1. Photography of albumen of two cultivars of coconut: yellow and green skin fruit.

antioxidant activity assay, the one-way ANOVA test was used, followed by the Tukey test ($P < 0.001$).

Apparatus/ Chemical analysis

The quantitative analysis of all the oil samples was performed in a Shimadzu GC-17A gas chromatography, using a dimethylpolysiloxane DB-5 fused silica capillary column (30m × 0.25mm, film thickness 0.25µm) and a flame ionization detector (FID). The H_2 was used as the carrier gas at a flow rate of 1mL/ min and with a 30psi inlet pressure; split 1:30; temperature program: 35 to 180 °C at 4 °C/ min, then heated at a rate of 17°C/ min to 280°C and held in isothermal for 10 min; injector temperature 250°C and the detector temperature was at 250°C. The fatty oils compositions were obtained from the GC/MS analysis, while the analysis of the samples were performed with a Hewlett-Packard 5971 GC/MS instrument, using the following conditions: dimethylpolysiloxane DB-1 fused silica capillary column (30m x 0.25mm, 0.1µm film thickness); carrier gas: He (1mL/ min); injector temperature: 250 °C; detector temperature: 200°C; column temperature: 35° to 180°C at 4°C/ min, then 180 to 250°C at 10°C/ min; mass spectra: electronic impact 70 eV. Each component was identified by two computers combined with library microsoft searches, using their retention indices followed by a visual inspection of the mass spectra from the literature (Adams, 2007) and the standards, NIST 98 and Wiley MS Libraries (Wiley 275). The retention index was calculated using reference, the retention times of a series in the standards for hydrocarbons (C11-C-28), under the same conditions, according to the calculations of the literature (Porte, 2000).

RESULTS AND DISCUSSION

The unripe oil of the green variety (1G), showed in the CG-MS analysis nine compounds as: 38.05% of carboxylic acids and 48.46% as hydrocarbons. The major compounds for this stage were methyl palmitate (19.23%); methyl elaidate (11.55%); isotetradecane (15.20%) and isohexadecane (14.37%). The unripe oil of the yellow variety (1Y) showed 67.32% of hydrocarbon and only 3.68% of the carboxylic acid. The major compounds for this stage were the isotetradecane (20.64%) and the isohexadecane (18.15%). In this particular stage, the coconut juice (1G) is used as a delicious soft drink, however, the juice from the yellow (1Y) coconut is not consumed, since the taste is not pleasant. The CG-MS analysis in the ripe oil from the green coconut (2G), showed 8.43% of hydrocarbons and 93.44% of the carboxylic acid, and the major compounds were the methyl laurate (38.27%); methyl mirystate (19.37%) and the methyl palmitate (12.38%). In the chemical composition of the ripe yellow oil (2Y), it was identified as having 4.62% of hydrocarbons and 68.26% of the carboxylic acid, when major compounds were identified as the same as the green variety in the ratio of 30.21, 12.34 and 9.00% respectively. The yield of oils and the presence of the carboxylic acid in this stage increased the composition with the unripe stage. At this point, the green coconut (2G) is used in the production of foods such as: cake, ice-cream and sweets. The dried stage of the green coconut (3G) is composed by 9.52% of hydrocarbons and 90.46% of the carboxylic acids, where the methyl laurate (57.80%) and the methyl mirystate (17.55%) were the major compounds. The yellow variety (3Y) yield with 9.39% of hydrocarbons and 88.72% of the carboxylic acids, were the major compounds and being the same of the green coconut and the ratios were 40.49 and 21.06% respectively. The yield of oils as well as the major constituents such as the lauric acid increased as the coconut became older. By now, the albumen of the coconut from the green variety (3G) is used with the extraction of oils and commercialized. The results, from the free radical scavenging effect with the fixed oil from the coconut of both cultivars, showed concentration-dependent activities. Concerning both cultivars, the free radical scavenging effect of the sample had the fixed oil of the ripe coconut and it was better compared to others stages (unripe and dried). The sample of the ripe stage showed significant antioxidant activity compared to the IC50 of Trolox and the BHT, which concludes that this composition may have increased the activity (Table 2).

Conclusion

Hydrocarbons, thioesters and the carboxylic acids were detected in the fixed oil of the two specimens with the solid albumen from the coconut in the three phases of maturity

Table 1. Chemical composition of coconut oils as methyl esters from the green (1G, 2G, 3G) and yellow (1Y, 2Y, 3Y) cultivars.

Compound	RT	Unripe (1G,1Y)		Ripe (2G, 2Y)		Dried (3G, 3Y)		Identification
		Green	Yellow	Green	Yellow	Green	Yellow	
Methyl hexanoate (methyl caproate)	3.10	-	-	-	0.90	-	0.24	RT, MS
Methyl octanoate (methyl caprilate)	6.25	-	-	2.10	1.20	1.98	4.51	RT, MS
Tridecane	10.40	5.96	8.41	0.18	0.10	-	-	RT, MS
Methyl decanoate (methyl caprinate)	10.96	-	-	3.87	2.42	7.54	4.64	RT, MS
2,6,11-trimetildodecane	12.34	4.20	-	-	-	-	-	RT, MS
Isotetradecane	12.91	15.20	20.64	-	-	-	-	RT, MS
2,8-dimetilundecane	14.47	8.73	11.51	-	-	-	-	RT, MS
Isohexadecane	15.40	14.37	18.15	-	-	-	-	RT, MS
Methyl dodecanoate (methyl laurate)	16.13	-	-	38.27	30.21	57.80	40.49	RT, MS
Dimyristyl tiodipropionate	20.12	3.95	-	-	-	-	-	RT, MS
n-heneicosane	20.13	-	6.28	-	-	-	-	RT, MS
Methyl tetradecanoate (methyl mirystate)	20.73	-	-	19.37	12.34	17.55	21.06	RT, MS
n-eicosane	23.64	-	2.33	-	-	-	-	RT, MS
Methyl hexadecanoate (methyl palmitate)	24.05	19.23	3.68	12.38	9.00	7.85	11.57	RT, MS
Methyl 9,12-octadecadienoate (methyl linoleate)	26.13	7.27	-	4.08	1.07	-	1.86	RT, MS
Methyl 9-octadecenoate (methyl elaidate)	26.23	11.55	-	16.37	7.90	4.90	8.44	RT, MS
Methyl ocadecanoate (methyl estereate)	26.50	-	-	2.97	3.12	2.36	5.30	RT, MS

RT – retention time; 1, 2, 3 - maturation stages unripe, ripe and dried; MS – mass spectrum.

Table 2. The DPPH free radical scavenging activity of oils samples[a,b]

Oil of coconut albumen	Concentration (µg/ml)								IC_{50} (µg/ml)
	0.1		0.001		0.00001		0.000001		
	Abs	Percent	Abs	Percent	Abs	Percent	Abs	Percentage (%)	
Unripe									
Green (1G)	0.0849±0.00005	68.9	0.1825±0.00003	33.2	0.2133±0.00002	21.9	0.2483±0.00003	9.1	5.2×10^{-3}
Yellow (1Y)	0.0937±0.00002	65.7	0.1992±0.00004	27.1	0.2240±0.00002	18.0	0.2532±0.00003	7.3	9.5×10^{-3}
Ripe									
Green (2G)	0.0713±0.00004	73.9	0.1571±0.00005	42.5	0.1882±0.00005	31.1	0.2314±0.00003	15.3	1.3×10^{-3}
Yellow (2Y)	0.0805±0.00003	70.5	0.1606±0.00004	41.2	0.1915±0.00002	29.9	0.2275±0.00005	16.7	1.7×10^{-3}
Dried									
Green (3G)	0.1377±0.00003	49.6	0.1912±0.00002	30.0	0.2221±0.00003	18.7	0.2478±0.00005	9.3	1.1×10^{-1}
Yellow (3Y)	0.1300±0.00003	52.3	0.1928±0.00006	29.4	0.2183±0.00003	20.1	0.2393±0.00005	12.4	5.9×10^{-2}
Trolox	0.0110±0.00003	96.0	0.0585±0.00002	78.6	0.1439±0.00002	47.3	0.2043±0.00005	25.2	1.9×10^{-5}
BHT	0.0052±0.00003	98.1	0.0538±0.00007	80.3	0.1210±0.00003	55.7	0.1909±0.00002	30.1	7.4×10^{-6}
Control	0.2732±0.00003	0.0	0.2732±0.00005	0.0	0.2732±0.00005	0.0	0.2732±0.00005	0.0	0.0

[a]The free radical scavenging effect was measured by the absorbance radical at 520 nm in a reaction containing the test sample and 60 µM DPPH; [b]Results are expressed as mean ± S.D.

duly analyzed therefore, are probably responsible in part, for the viscosity and odor of this particular oil. Furthermore, the palmitic acid has been present and increasing the concentration of each maturity phase for the skin yellow coconut, on the flip side, the palmitic acid has been present and decreasing the concentration of each maturity phase for the skin green coconut, probably it is a compound common to all. It was also observed in relation to the maturity, that a lot of the green species especially the yellow type had the amount of fixed oil increased, in other words, the composition was richer in the fat acids. In addition, it was observed as well that when it is unripe their majority constituents are formed by ramified hydrocarbons, which means that they can probably be oxidized in the following carboxylic acids when becoming ripe or dried. A high scavenging activity was found in samples obtained from the ripe coconut and a moderate activity was observed in the unripe and the dry samples. The authors of this study suggest that the data from the antioxidant activities in the coconut oil, can be used as a natural antioxidant additive.

ACKNOWLEDGMENT

This study was supported by the funds and fellowships from the following Brazilian Government Agencies: CNPq, FUNCAP, CAPES and FAPESB.

Conflict of Interest

We declare that we have no conflict of competing interest.

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