Journal of
Medicinal Plant Research
Volume 11 Number 10, 10 March, 2017
ISSN 1996-0875

Academic Journals

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Journal of Medicinal Plants Research

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Vol. 11(10), pp. 194-206, 10 March, 2017
DOI: 10.5897/JMPR2016.6320
Article Number: 6C4932063204
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

Pharmacological potential of *Palicourea rigida* kunth: A possible participation of flavonoid compounds

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Received 15 December, 2016: Accepted 6 March, 2017

Palicourea rigida **Kunth (Rubiaceae), also called "bate-caixa" or "douradão", has been used as antihypertensive, antiulcerogenic, anti-inflammatory and analgesic by traditional communities. Pharmacological potential of the ethanol extract from *P. rigida* (EEPR) and two quercetin derivatives were investigated. Using the high performance liquid chromatography (HPLC) assay, EEPR was analyzed. Phenolic contents (total phenolic and flavonoids) were quantified by spectrophotometric methods. 2,2-diphenyl-1-pycrilhydrazil (DPPH), iron reducing power and β-carotene/linoleic acid bleaching tests were applied to estimate the antoxidant capacity of EEPR. Nociception (acetic acid-induced writhing, formalin and hot plate) and inflammation (carrageenan-induced paw edema and pleurisy) assays were performed. Molecular docking was used to measure the interactions' profiles of ligands (rutin and quercetin) and cyclooxigenases (COX-1 and COX-2). HPLC analysis identified rutin and quercetin derivatives. Expressive levels of total phenolic and flavonoids and a promising antioxidant effect were measured. EEPR, rutin and quercetin reduced the abdominal contortions. EEPR was effective against both phases of formalin, while rutin and quercetin inhibited the second phase. The latency time on hot plate significantly increased after treatment with EEPR. Inflammatory parameters (paw edema, exudate volume and leukocyte infiltrate) were diminished by EEPR, rutin and quercetin. The molecular docking showed that rutin and quercetin are capable of complexing with COX-1 and COX-2 favorably through physical-chemical interactions. The results suggest that EEPR showed a relevant pharmacological potential, which may be related to action of rutin and quercetin derivatives.**

Key words: *Palicourea rigida*, rutin, quercetin, antioxidant, antinociception, inflammation.

INTRODUCTION

In the body, the imbalance between oxidant/antioxidant in favor of the oxidation promotes the oxidative stress that causes proteins, lipids and DNA damages, as well as induces a variety of cellular responses through the generation of reactive oxygen species (ROS) that may be originated from cellular metabolism or environmental sources (Nita and Grzybowski, 2016). ROS have become a concern among researchers because they have been

associated with a significant number of diseases involving inflammatory and painful processes that affect humans and animals. In inflammation, for example, ROS cause vascular damage which allows the permeability of macromolecules and inflammatory cells from the blood to tissue (Mittal et al., 2014). This permeability is controlled by vasoactive and chemotactic mediators, which make the inflammatory process active, including the pain (Silva, 2015). Among the mediators that regulate the events of inflammation, vasoactive amines, lipid-derived eicosanoids, cytokines, chemokines and adhesion molecules have been highlighted (Silva, 2015). On the other hand, the inflammatory pain has been treated with non-steroidal anti-inflammatory agents, but present high prevalence among adverse reactions to drugs (Derle et al., 2006). However, natural products, as flavonoids, have been investigated for their mechanisms against painful, inflammatory and oxidative processes (Agrawal, 2011; Iwalewa et al., 2007).

Palicourea is a plant genus in the family Rubiaceae and contains about 200 species (Rosa et al., 2010). Plants of this genus, as *Palicourea rigida* Kunth, are distributed in the Tropics of the New World, particularly in the South America' Brazilian Cerrado region (Morel et al., 2011). *P. rigida*, commonly known as "douradinha", "bate-caixa" and "douradão" and the medicinal uses as antifungal, analgesic, diuretic, hypotensive, antiulcerogenic, cicatrizing and anti-inflammatory have been made by traditional communities (Rosa et al., 2010). From the chemical view-point, triterpenes, flavonoids, iridoids, alkaloids and peptides have been identified in this medicinal plant (Rosa et al., 2010; Morel et al., 2011; Pinto et al., 2012; Soares et al., 2012). Antioxidant (Rosa et al., 2010), antibacterial and insecticide (Pinto et al., 2012) and antiproliferative (Soares et al., 2012) activities have been related to these compounds.

Additionally, rutin is a flavone glycoside widely known and its hydrolysis produces quercetin and rutinose by the action of glucosidase (Chua, 2013). Both rutin and quercetin are found in medicinal plants, and anti-inflammatory (Chua, 2013; Choi et al., 2012), anti-tumor (Ren et al., 2003), anti-asthma (Jung et al., 2007), and antioxidant (Yang et al., 2008) activities are attributed to these compounds. Beside these data, quercetin 3-O-D-glucoside, quercetin 3-O-sophoroside and isorhamnetin 3-glucoside were identified in *P. rigida* (Rosa et al., 2010), which can justify the biological properties of this plant.

Considering that the scientific evidences of medicinal plants are fundamental for the therapeutic use, this article was described to investigate the antioxidant, antinociceptive and anti-inflammatory activities of EEPR using *in vitro*, *in vivo* and *in silico* tools. In addition, due to the chemical characterization and phenolic and flavonoid contents of EEPR, two markers (rutin and quercetin) were evaluated in order to establish possible bioactive compounds and mechanism of action.

MATERIALS AND METHODS

Plant material and extraction

P. rigida leaves were collected in the city of São João del-Rei, Minas Gerais State, Southeast region of Brazil, in May 2010. The species was identified by Dr. Glauciemar Del-Vechio Vieira and registered in the Herbarium of the Department of Botany, Federal University of Juiz de Fora, Brazil, under number CESJ 42.677. After drying, 850 g of powdered leaves were subjected to extraction by static maceration in 95% ethanol (2.5 L) to obtain the ethanol extract (EEPR) through filtration. Extractive solution was evaporated (rotary evaporator, R-215 Büchi Labortechnik AG, Flawil, Switzerland) at 50 to 60°C. After removal of the water and solvent in a desiccator, the yield was of 66.68 g.

Chemicals

In this study, the following chemicals were used: Acetic acid, acetylsalicylic acid and aluminum chloride (Vetec Química Fina Ltda, Sigma-Aldrich Corporation, Rio de Janeiro, RJ, Brazil), formaldehyde (Quimibrás Indústria Química S/A, Rio de Janeiro, RJ, Brazil), Folin-Ciocalteu reagent, trichloroacetic acid, and ascorbic acid (Cromoline Química Fina, Diadema, SP, Brazil), potassium ferrocyanide, ferric chloride, methanol, ethanol, pyridine and sodium carbonate (Labsynth, Diadema, SP, Brazil), morphine hydrochloride (Merck Inc., Whitehouse Station, NJ, USA), naloxone and indomethacin (Sigma Chemical Co, St Louis, MO, USA) and DPPH, linoleic acid, β-carotene, tween® 40, galic acid, BHT, rutin, quercetin, kaempferol, luteolin, luteolin 7-*O*-β-D-glucoside, apigenin and apigenin 7-O-β-D-glucoside (Sigma-Aldrich Chemie, Buchs, SG, Switzerland).

Animals

In this experiment, *Mus musculus* L. (male Swiss albino mice, 50-70 days; 25-30 g) and *Rattus norvegicus albinus* (male Wistar rats, 90-110 days; 200-240 g) were supplied by the Central Biotery of the Federal University of Juiz de Fora (UFJF). Groups of animals were maintained in plastic cages (47 × 34 × 18 cm^3) under a 12 h light/12 h dark cycle at room temperature (22 ± 2°C), with free access to rations (Nuvilab Rodents - Nuvital Nutrients, Colombo, Brazil) and water. The protocols (047/2012 and 049/2012) were approved by the ethical committee of UFJF, which are in accordance with the guidelines recommended by the Brazilian College of Animal Experimentation (COBEA).

Phytochemical screening

Preliminary phytochemical analysis of EEPR was determined by the following procedures (Tiwari et al., 2011).

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Tannins

Gelatin test - To EEPR, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

Flavonoids

Alkaline reagent test: EEPR was treated with few drops of sodium hydroxide solution. Formation of intense yellow colour, which becomes colourless on addition of dilute acid, indicates the presence of flavonoids.

Lead acetate test: EEPR was treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.

Diterpenes

Copper acetate test: EEPR was dissolved in water and treated with 3 to 4 drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

Phytosterols

Libermann Burchard's test: EEPR was treated with chloroform and filtered. The filtrate was treated with few drops of acetic anhydride followed by boiled and cooled with addition of sulphuric acid. The positive reaction was observed through a brown ring at the junction.

Saponins

Foam test: 0.5 mg of EEPR with 2 ml of water was agitated in test tubes. Foaming for 10 min, it indicates positive reaction to saponins.

Coumarins

5 ml of EEPR was evaporated; the residue was dissolved in 1 to 2 ml of hot distilled water and the volume was divided into two parts. Half of the volume was taken as a witness and another volume of 0.5 ml 10% NH_4OH was added. Two spots were placed on filter paper and examined under UV light. Intense fluorescence indicates the presence of coumarins.

Anthraquinones

Modified Borntrager's test: EEPR was treated with ferric chloride solution and immersed in boiling water for around 5 min. The mixture was cooled and extracted with equal volume of benzene. The benzene layer was separated and treated with ammonia solution. Formation of rose-pink colour in the ammonical layer indicates the presence of anthranol glycosides.

Alkaloids

EEPR was dissolved in diluted hydrochloric acid and filtered.

Mayer's test: Filtrated was treated with Mayer's reagent (potassium mercuric iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Dragendroff's test: Filtrated was treated with Dragendroff's reagent (potassium bismuth iodide solution). Formation of red precipitate indicates the presence of alkaloids.

Total phenolic determination

To quantify the total phenolic, spectrophotometric method was applied using the Folin-Ciocalteu reagent (Sousa et al., 2007). Concentrations of EEPR (400, 800 and 1200 µg/mL) were prepared for this determination. The calibration curve was established with gallic acid (200 to 760 µg/mL) and the obtained absorbances were submitted to linear regression analysis using the least squares method to acquire the equation of the line and the correlation coefficient (r). In this reaction, the Folin-Ciocalteu reagent is capable of oxidising phenolic constituents and the neutralization is done by sodium carbonate with generation of a blue staining. After 60 min of reaction, the absorbance was recorded at 765 nm in spectrophotometer (Shimadzu®, UV-1800, Tokyo, Japan). All analysis were carried out in triplicate and the average shown as gram of gallic acid equivalent (g/100 g).

Total flavonoids determination

Spectrophotometric method was performed for total flavonoid determination using rutin as standard (Sobrinho et al., 2008). For this quantification, concentrations of EEPR (400, 800 and 1200 µg/mL) were prepared. The calibration curve was elaborated with rutin (2 to 60 µg/mL) in $AlCl_3$ (8% in ethanol) and the obtained absorbances were submitted to linear regression analysis using the least squares method to acquire the equation of the line and the correlation coefficient (r). In this procedure, aluminum chloride reacts with flavonoids of EEPR in the presence of acetic acid, pyridine:ethanol (2:8) and distilled water at room temperature for 30 min. After this time, the absorbances, in triplicate, were determined at 420 nm using a spectrophotometer (Shimadzu®, UV-1800, Tokyo, Japan). The results were demonstrated as gram of rutin equivalent (g/100 g).

High pressure liquid chromatography (HPLC) analysis

The methodology used in this analysis was described by Silva et al. (2013) previously validated by the Laboratory of Natural Products/Institute of Biological Sciences/UFJF. The HPLC system consisted of an Agilent Technologies 1200 Series with a PDA detector and an automatic injector. The column employed was a Zorbax SB-18; 250 × 4.6 mm, 5 µm particle size. Solvents that constituted the mobile phase were A (water pH adjusted to 4.0 with H_3PO_4) and B (acetonitrile). The elution conditions applied were: 0-30 min, 20% B isocratic. The mobile phase was returned to the original composition over the course of 30 min, and an additional 5 min were allowed for the column to re-equilibrate before injection of the next sample. The sample volume was 50 µl at a concentration of 1 mg/mL, the flow rate of 0.6 mL/min and the temperature was maintained at 25°C during the analysis. Detection was performed at 254 nm. Gallic acid, rutin, quercetin, kaempferol, luteolin, luteolin 7-*O*-β-D-glucoside, apigenin and apigenin 7-*O*-β-D-glucoside were also used as possible markers.

DPPH radical scavenging activity

In this experiment, using DPPH method, the antioxidant activity was measured (Mensor et al., 2001). EEPR solutions (1 to 100 µg/mL)

were prepared and mixed with methanol solution of DPPH (0.03 mM). Using a spectrophotometer (Shimadzu®, UV-1800, Tokyo, Japan), the absorbance values were measured at 518 nm after 60 min kept at 22 ± 2°C. The experiment was performed in triplicate. Rutin, quercetin and ascorbic acid were used as references. After obtaining the absorbances (Abs) of the samples, blank and control, the percentage of antioxidant activity (%AA) was determined using the following equation:

$$\%AA = \frac{100 - [(Abs\ sample - Abs\ blank) \times 100]}{Abs\ control}$$

The 50% effective concentration (EC_{50}) of EEPR was obtained by linear regression analysis using the least squares method to acquire the equation of the line and the correlation coefficient (r). Half maximum effective concentration (EC_{50}) denotes the concentration (µg/mL) of EEPR required to reduce 50% of DPPH.

Antioxidant activity by reducing power

According to the method recommended by Oyaizu (1986), the antioxidant activity by reducing power was evaluated. EEPR solutions (750 to 100 µg/mL) reacted with 1% potassium ferrocyanide (in 0.2 mM phosphate buffer, pH 6.6) and kept at 50ºC for twenty minutes. After this time, 10% TCA (trichloroacetic acid) was mixed and centrifuged (3000 g over 10 minutes) to separate the supernatant. Distilled water containing 1% ferric chloride was mixed with the supernatant and the absorbance values, in triplicate, were recorded at 700 nm by spectrophotometry (Shimadzu®, UV-1800, Tokyo, Japan). Rutin, quercetin and ascorbic acid were used as references. EC_{50} was calculated from the graph of sample concentrations (X axis) and absorbances (Y axis) submitted to linear regression analysis using the least squares method to acquire the equation of the line and the correlation coefficient (r). The effective concentration (EC_{50}) was determined in the absorbance of 0.5.

Antioxidant activity by lipid peroxidation method

Using the spectrophotometric method described by Miller (1971) with some modifications, the β-carotene bleaching test was performed with 0.2 mg/mL β-carotene (1 mL diluted in chloroform), linoleic acid (0.02 mL) and Tween 20 (0.2 mL). Then, the chloroform was evaporated (rotary evaporator, R-215 Büchi Labortechnik AG, Flawil, Switzerland), the mixture was previously oxygenated for 30 min with addition of distilled water to produce an emulsion. EEPR, rutin and quercetin (38.46 to 1.20 µg/mL) were placed in test tubes containing 5 mL of emulsion, which were inserted in water bath (50°C for 2 h). After this procedure, absorbances were determined spectrophotometrically (spectrophotometer Shimadzu®, UV-1800, Tokyo, Japan) in zero, 15, 30, 45, 60, 75, 90 and 105 min at 470 nm, in triplicate. BHT was used as standard. The percentage of inhibition of lipid peroxidation (%) was calculated.

Acute toxicity

To define the doses that were administered in male mice in the study of antinociceptive activity described below, the acute toxicity was evaluated using this animal gender. To perform this procedure, mice (n = 10) were orally (per oral route, p.o.) treated with doses of EEPR (0.5 to 3 g/kg) and saline (control group). The toxicity was also investigated by signs and symptoms and the number of death was totalized for 48 h. The probit test proposed by Litchfield and Wilcoxon (1949) was used to determine the LD_{50} (50% lethal dose). Based on the description of the pharmacological activity studies, the highest dose (400 mg/kg) is less than 500 mg/kg of acute toxicity, which may justify the doses chosen. Additionally, because rutin and quercetin are pharmaceutical and nutraceutical used by the population, their acute toxicity was not investigated.

Acetic acid-induced chemical nociception

Considering the description on acute toxicity assay, animal groups (n = 8) were treated with EEPR (100 to 400 mg/kg, p.o.), rutin (400 mg/kg, p.o.), quercetin (400 mg/kg, p.o.), acetylsalicylic acid (200 mg/kg, p.o.), indomethacin (10 mg/kg, p.o.) and saline orally (10 mL/kg, p.o.) (Collier et al., 1968). One hour after treatment, 0.6% acetic acid (0.1 mL/10 g, i.p.) was applied intraperitoneally (i.p.). The abdominal writhes were measured between 10 and 30 min after application of acetic acid.

Formalin-induced chemical nociception

The experiment was conducted according to Hunskaar and Hole´s (1987) method using animal groups (n = 8). The treatment of the mice was performed with saline (10 mL/kg, p.o., negative control), EEPR (100 to 400 mg/kg, p.o.), rutin (400 mg/kg, p.o.), quercetin (400 mg/kg, p.o.), indomethacin (10 mg/kg, p.o., positive control) or morphine (5 mg/kg, s.c., positive control), one hour before formalin injection. After injection of 2.5% formalin (20 µL, in sterile saline) in the subplantar right hind paw region, the licking times of the neurogenic (0–5 min) and inflammatory phases (15-30 min) were evaluated.

Hot plate-induced thermal nociception

As recommended by Eddy and Leimbach (1953), mice (n = 8) were previously treated with EEPR (100 to 400 mg/kg, p.o.), rutin (400 mg/kg, p.o.), quercetin (400 mg/kg, p.o.), saline (10 mL/kg, p.o., negative control) and morphine (5 mg/kg, s.c., positive control). Then, the animals were placed on a hot plate (Model LE 7406, Letica Scientific Instruments, Barcelona, Spain) at 55 ± 1°C and the reaction time (s) was determined at 0, 30, 60 and 90 min. The maximum permanence time on the hot plate was 30 s. In separate groups, naloxone (2 mg/kg, s.c.), an opioid antagonist, was used in presence of EEPR (400 mg/kg) and morphine (5 mg/kg, s.c.) to investigate the central action mechanism.

Carrageenan-induced paw edema

Anti-inflammatory activity was evaluated by carrageenan-induced paw edema method in Wistar rats (Winter et al., 1962). In this assay, the animals were treated with EEPR (100 to 400 mg/kg, p.o.), rutin (400 mg/kg, p.o.), quercetin (400 mg/kg, p.o.), indomethacin (10 mg/kg, p.o., positive control) and saline (10 mL/kg, p.o., negative control). After 1 h of treatment, the rats were previously anesthetized with ketamine (60 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.) solution and 1% carrageenan (0.1 mL) was administered into the right paw, while the left paw was injected with saline (0.1 mL). Using a plethysmometer (model LE 7500, Letica Scientific Instruments, Barcelona, Spain), the paw edema was measured at 1, 2, 3 and 4 h after carrageenan injection by differences between the paws (right and left).

Carrageenan-induced pleurisy

According to Vinegar et al. (1973) with minor modifications, rats (n

Table 1. Chemical contents and antioxidant activity of EEPR.

Extract/ chemical	Contents (g/100 g)		EC_{50} (µg/mL)		Inhibition of lipid peroxidation (%)
	Total phenolic	Total flavonoid	DPPH	Fe^{+3} reducing power	
EEPR	5.78±0.05	3.95±0.02	65.37±0.16	697.36±2.48	64.46±1.34
Rutin	-	-	8.72±0.35	212.93±4.34	24.30±3.38
Quercetin	-	-	22.16±0.24	119.27±0.74	53.09±2.38
Ascorbic acid	-	-	12.41±0.30	5.84±0.03	-
BHT	-	-	-	-	84.76±2.80

Values are mean ± S.E.M. (n = 3). EC_{50} and inhibition of lipid peroxidation column values were different after ANOVA followed Tukey test (p < 0.05). EEPR: Ethanol extract from *P. rigida*; BHT: Butylated hydroxytoluene.

= 6) were treated with EEPR (100 to 400 mg/kg, p.o.), rutin (400 mg/kg, p.o.), quercetin (400 mg/kg, p.o.), saline (10 mL/kg, p.o.) and indomethacin (10 mg/kg, p.o.). One hour after treatment, the animals were intraperitoneally anesthetized with ketamine and xylazine (60 and 8 mg/kg, respectively), and a suspension of saline containing 1% carrageenan (0.4 mL) was injected into the pleural cavity. After 4 h of pleurisy, overdose of anesthetic solution (120 mg/kg of ketamine and 16 mg/kg of xylazine 16 mg/kg, i.p.) was applied to induce the euthanasia of the animals. The pleural exudate was collected in the lung cavity to determine the volume and total leucocyte.

Evaluation of the molecular interaction profiles

The ligands (rutin, quercetin, acetylsalicylic acid and indomethacin) were generated in Marvin Sketch Program and refined by the semi-empirical method PM7 presents in the MOPAC2012 Program. The inflammatory enzymes were obtained from Protein Data Bank under the 1EQG (COX-1) and 5IKT (COX-2) codes. Subsequently, the Gasteiger's loads were determined for all ligands and receptors through MGLTools Program. The molecular recognition was defined using the Discovery Studio v 4.5 2016 Program.

Statistical calculations

Mean and standard error of mean (S.E.M.) were obtained by statistical analysis. The level of significance (p < 0.05) was determined by one-way analysis of variance (ANOVA) followed by the Kruskal-Wallis or Tukey tests. For a better statistical determination, the Graph Pad® Prism 5.0. software was used.

RESULTS

Chemical determination and antioxidant activity

Using specific reagents, tannins, flavonoids, coumarins, terpenoids and steroids, saponins, anthraquinones and alkaloids were detected in EEPR.

EEPR revealed total phenolic and flavonoid contents and antioxidant activity (Table 1). In this extract, the total phenolic content was of 5.78±0.05 g/100 g, while the flavonoid reached 3.95±0.02 g/100 g. EEPR produced EC_{50} equal to 65.37±0.16 µg/mL (DPPH) and 697.36±2.48 µg/mL (reducing power). Using β-carotene bleaching assay, the inhibition of lipid peroxidation of EEPR was 64.46%. As expected, rutin and quercetin showed antioxidant effect in these methods. In addition, quercetin was more active than rutin (a glycosylated flavonoid) in lipid peroxidation, since such assay is related to the oxidative stress in biological membranes.

High pressure liquid chromatography (HPLC) analysis

Considering the UV spectrum and the retention time of the main peaks, rutin (peak 13, t_R = 30.72 min) was identified (Figure 1). However, rutin content was not quantified. The peak 12 represented a quercetin derivative. Although gallic acid, kaempferol, luteolin, luteolin 7-*O*-β-D-glucoside, apigenin and apigenin 7-O-β-D-glucoside had been reported in other species of *Palicourea*, using the conditions described above, these markers were not detected in EEPR. It is possible that flavonoids, together with other compounds, have a synergistic action that may justify the pharmacological effects of *P. rigida*.

Assessment of the acute toxicity

EEPR revealed no toxicity to treated animals, since produced LD_{50} up to 3000 mg/kg. After 48 h of treatment, the animals showed no signs or symptoms of toxicity. The LD_{50} value was important in defining the pharmacologic doses.

Effect on acetic acid-induced chemical nociception in mice

When compared to the control group, 100, 200 and 400 mg/kg of EEPR significantly (p < 0.05) reduced the writhes in 32.92, 33.62 and 62.26%, respectively. Rutin (24.36%) and quercetin (25.39%) also diminished the abdominal contortions (p < 0.05). Indomethacin (75.64%) and acetylsalicylic acid (69.22%) proved to be efficient as analgesic agents, since were able to inhibit the abdominal

Table 2. Effects of EEPR, rutin and quercetin on the reaction time of the hot plate in mice.

Group	Dose (mg/kg)	Time after drug administration (s)			
		0 min	30 min	60 min	90 min
Control	Saline	5.45±0.46	5.60±0.37	5.79±0.29	6.01±0.25
EEPR	100	5.46±0.38	5.64±0.31	6.22±0.32	6.23±0.33
	200	5.55±0.31	5.81±0.30	7.09±0.26	8.21±0.18*
	400	5.54±0.27	6.75±0.33	8.70±0.44*	10.29±0.49*
Rutin	400	5.33±0.25	5.47±0.62	5.70±0.27	5.98±0.34
Quercetin	400	5.12±0.27	5.46±0.34	6.03±0.29	6.10±0.34
Morphine	5	5.48±0.38	7.42±0.25*	10.57±0.56*	13.62±0.71*
Naloxone+morphine	2+5	5.50±0.37	7.10±0.23*	6.97±0.30	6.87±0.27
Naloxone+Extract	2+400	5.54±0.38	6.69±0.21	7.29±0.30*	7.53±0.18*

Values are mean ± S.E.M. (n = 8). *p < 0.05 represents the significance level when compared with negative control group after ANOVA followed by Kruskal-Wallis test. EEPR: Ethanol extract from *P. rigida*

contortions (Figure 2).

Effect on formalin-induced chemical nociception in mice

The duration of paw licking for the control group was 74.25 ± 2.60 s (first phase) and 86.75 ± 2.40 s (second phase). EEPR (200 and 400 mg/kg) and morphine (5 mg/kg) significantly reduced the paw licking time in the nociceptive phase (Figure 3). In this phase, rutin and quercetin were not actives. The licking time was reduced after treatment with EEPR (100 to 400 mg/kg), rutin and quercetin in the second phase (p < 0.05). Even by different mechanism, indomethacin (cyclooxygenase inhibitor) and morphine (opioid agonist) were highly effective in decreasing the late phase.

Effects on hot plate-induced thermal nociception in mice

To evaluate the possible central effect indicated in the formalin test, the hot plate-induced thermal nociception method was used in this investigation. After one hour of treatment, EEPR (400 mg/kg) increased the reaction time of the thermal stimulation when compared to the control (p < 0.05). Doses of 200 (p < 0.05) and 400 mg/kg (p < 0.05) also increased the reaction time after 90 min (Table 2). In this experiment, rutin and quercetin produced no significant activity. As expected, morphine increased the reaction time and naloxone blocked this effect. Naloxone was not able to completely antagonize the effect of EEPR.

Effects on paw edema model

EEPR (400 mg/kg, 19.70%; p < 0.05) reduced the paw edema from 2 h after carrageenan application (Figure 4). This effect was observed after 3 h at the doses of 200 and 400 mg/kg of EEPR, as well as rutin and quercetin (p < 0.05). After 4 h carrageenan application, 200 and 400 mg/kg of EEPR, rutin and quercetin (p < 0.05) also showed significant effect. At this time, indomethacin reduced the paw edema by 25.92%.

Effects on pleurisy model

The results of the paw edema were confirmed by the pleurisy model (Table 3). In the Table 3, considering the control group, doses of 200 (27.18%) and 400 mg/kg (36.89%) of EEPR significantly (p < 0.05) reduced the exudate volume. These doses (200 and 400 mg/kg) also decreased the number of total leukocytes (p < 0.05) in 11.94 and 24.91%, respectively (Table 3). Rutin, quercetin and indomethacin (positive control) reduced the inflammatory parameters.

Molecular interaction profiles by docking

The molecular docking study showed that the ligands (rutin and quercetin) are capable of complexing with cyclooxygenase-1 (rutin = -9.1 $Kcalmol^{-1}$; quercetin = -9.6 $Kcalmol^{-1}$) and cyclooxygenase-2 (rutin = -9.8 $Kcalmol^{-1}$; quercetin = -8.2 $Kcalmol^{-1}$) more favorably than acetylsalicylic acid (-7.0 and -7.3 $kcalmol^{-1}$ to COX-1 and COX-2, respectively) and indomethacin (-6.3 and -6.8 $kcalmol^{-1}$ to COX-1 and COX-2, respectively) (Table 4). These data indicated that rutin and quercetin have higher affinity for the site of action of these enzymes than the reference compounds. In addition, van der Waals, dipole-dipole and hydrogen bonding interactions were recognized by the inflammatory enzymes (COX-1 and COX-2) (Figures 5 and 6).

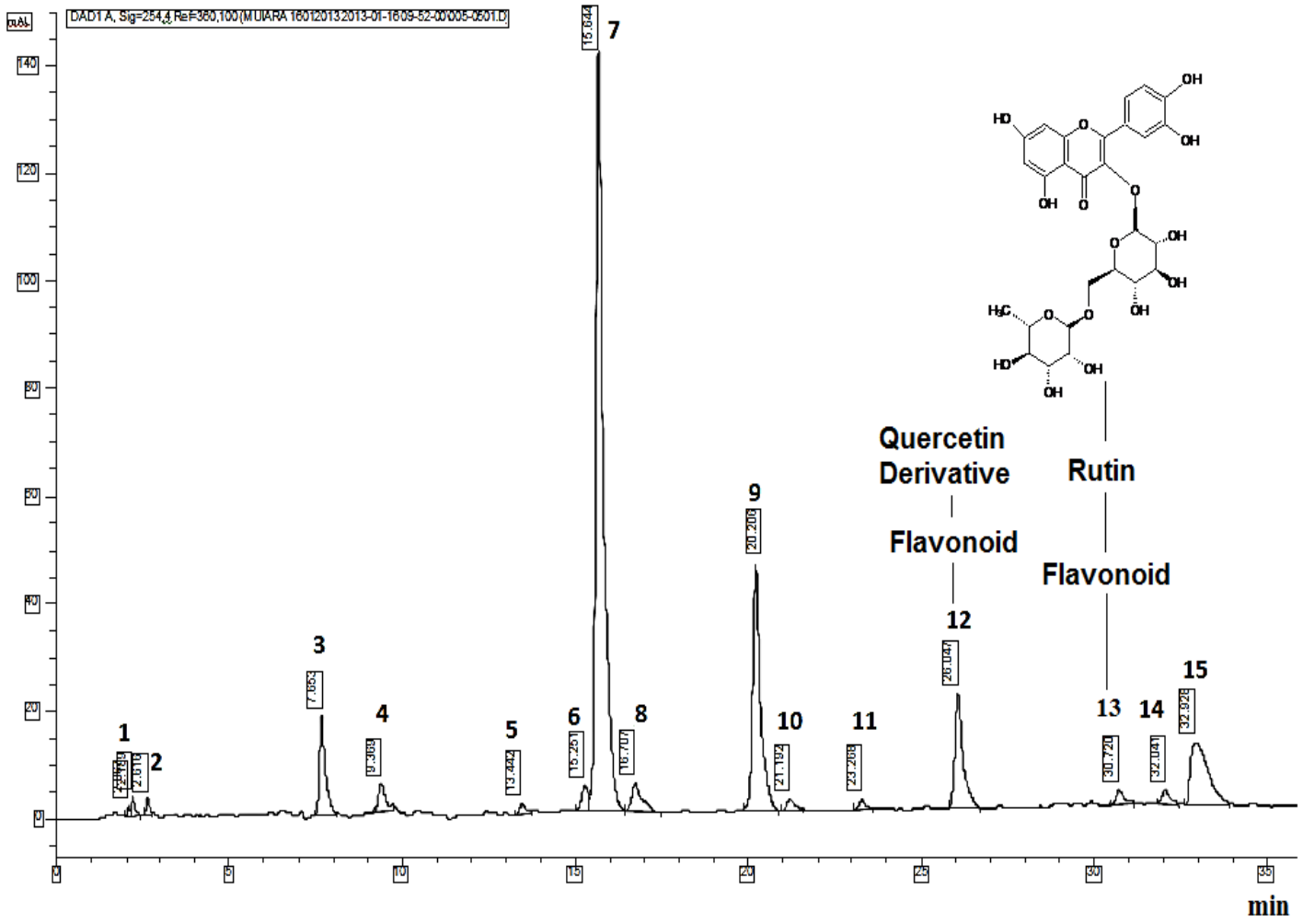

Figure 1. HPLC analysis of EEPR showing the presence of rutin (peak 13) at 254 nm.

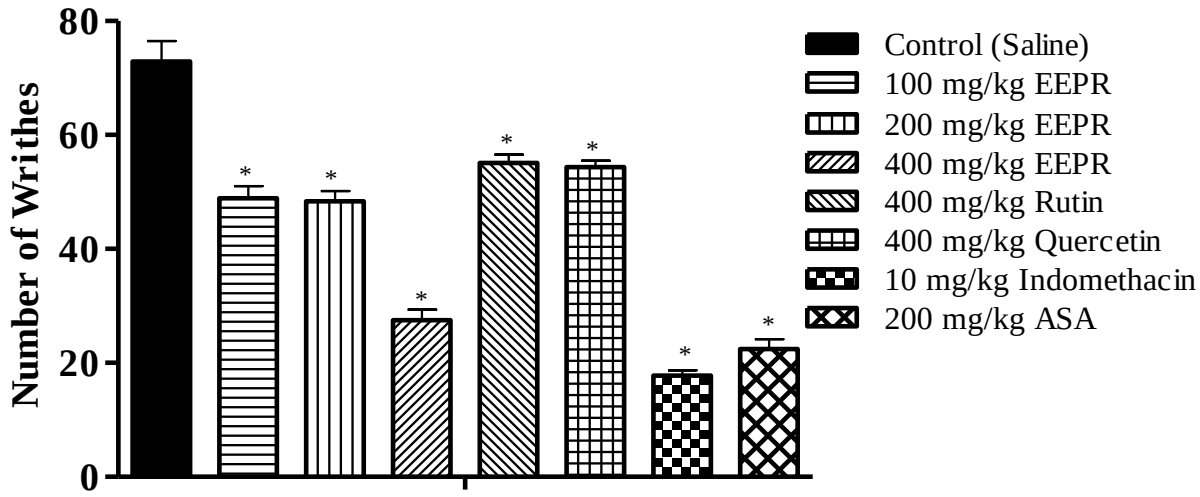

Figure 2. Effects of EEPR, rutin and quercetin on acetic acid-induced nociception. Values are mean ± S.E.M. (n = 8). *p < 0.05 represents the significance level when compared with negative control group after ANOVA followed by Kruskal-Wallis test. EEPR: Ethanol extract from *P. rigida*. ASA: acetylsalicylic acid.

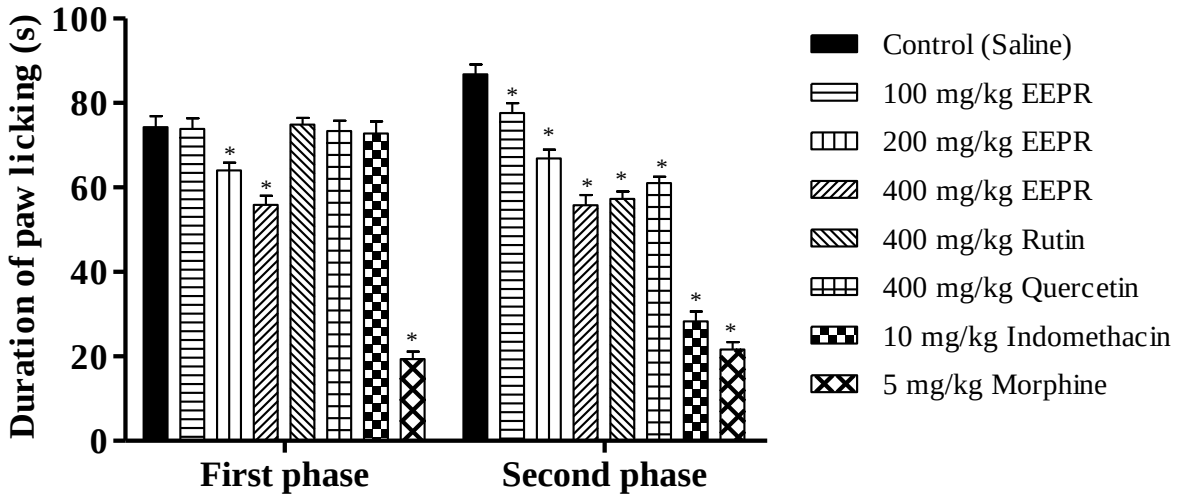

Figure 3. Effects of EEPR, rutin and quercetin on formalin-induced nociception in mice. Values are mean ± S.E.M. (n = 8). *p < 0.05 represents the significance level when compared with negative control group after ANOVA followed by Kruskal-Wallis test. EEPR: Ethanol extract from *P. rigida*. ASA: acetylsalicylic acid. First phase (0-5 min) and second phase (15-30 min).

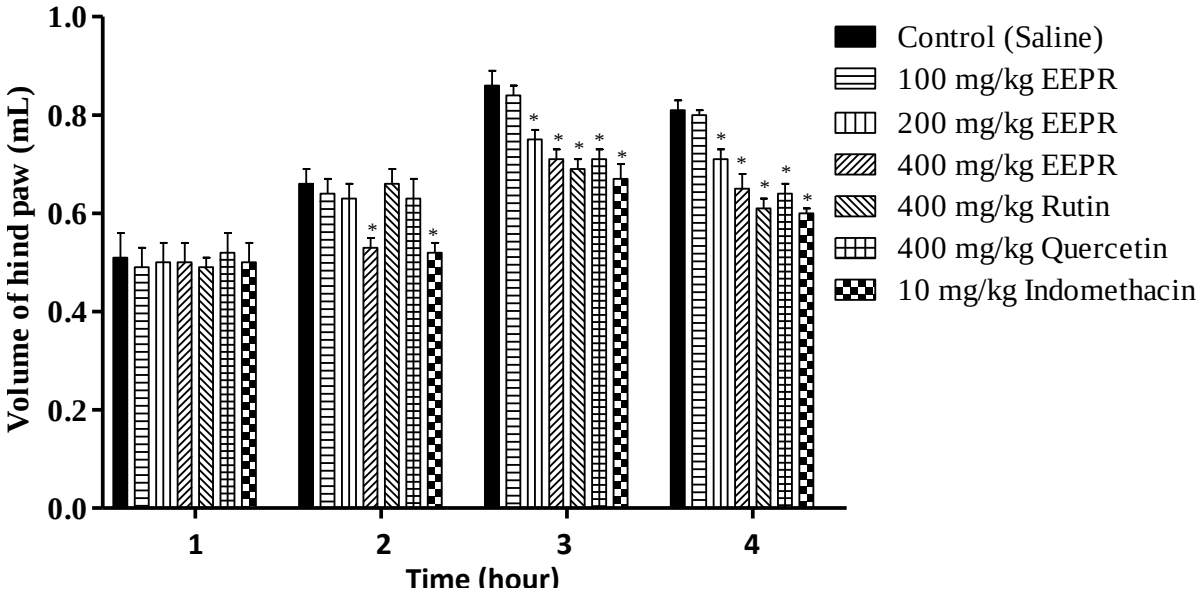

Figure 4. Effects of EEPR, rutin and quercetin on paw edema. Values are mean ± S.E.M. (n = 6). *p < 0.05 represents the significance level when compared with negative control group after ANOVA followed by Kruskal-Wallis test. EEPR: Ethanol extract from *P. rigida*.

DISCUSSION

Plants containing flavonoids have been associated with different medicinal uses such as diuretic, laxative, antispasmodic, anti-hypertensive, antinociceptive, and anti-inflammatory (Agrawal, 2011). The HPLC analysis of EEPR identified rutin, which has been reported as antioxidant, anti-inflammatory, antiasthmatic, and analgesic agents (Azevedo et al., 2013), and our results are in concordance with this description. In addition, flavonoids (quercetin 3-O-D-glucoside, quercetin 3-O-sophoroside and isorhamnetin 3-glucoside) were also isolated and identified in *P. rigida* (Rosa et al., 2010) and these compounds are well known for their ability to inhibit oxidative, painful and inflammatory mechanisms (Agrawal, 2011; Azevedo et al., 2013). Prostaglandins, for example, are related to generation of immunological mediators and are produced by the cyclooxygenase pathway, which can

Table 3. Effects of EEPR, rutin and quercetin on inflammatory parameters of pleurisy in rats.

Group	Dose (mg/kg)	Exsudate volume (mL)	Inhibition (%)	N° Leukocytes × (10^3 cells/mm^3)	Inhibition (%)
Control	Saline	1.03±0.08	-	13.73±0.26	-
	100	0.98±0.06	4.85	13.27±0.25	3.35
EEPR	200	0.75±0.04*	27.18	12.09±0.27*	11.94
	400	0.65±0.04*	36.89	10.31±0.28*	24.91
Rutin	400	0.71±0.03*	31.07	11.70±0.17*	14.78
Quercetin	400	0.72±0.03*	30.09	11.67±0.24*	15.00
Indomethacin	10	0.58±0.05*	43.69	9.07±0.25*	33.94

Values are mean ± S.E.M. (n = 6). *p < 0.05 represents the significance level when compared with negative control group after ANOVA followed by Kruskal-Wallis test. EEPR: Ethanol extract from *P. rigida*

Table 4. Binding affinity of the molecular interactions between ligands and cyclooxygenases.

Ligands	Binding affinity (Kcal/mol)	
	Cyclooxygenase-1	Cyclooxygenase-2
Rutin	-9.1	-9.8
Quercetin	-9.6	-8.2
Indomethacin	-6.3	-6.8
Acetylsalicylic acid	-7.0	-7.3

be inhibited by natural substances as flavonoids (Agrawal, 2011). Thus, based on our data, EEPR flavonoids, mainly rutin and quercetin tested in the current investigation, contributed for the antioxidant, antinociceptive and anti-inflammatory activities, since these compounds were effective in the applied tests.

Based on the literature, the flavonoid content has not been previously reported for *P. rigida* and the total phenolic content seems to be higher than that described by Rosa et al. (2010). Phenolic compounds and other natural products are known for their antioxidant action, since they are capable of donate electrons to free radicals. Using DPPH method, Rosa et al. (2001) showed an EC_{50} value eight times smaller than our result and associated the antioxidant effect with to the presence of phenolic compounds (total phenolic) in *P. rigida* that exhibit mechanism against this radical. In addition, the antioxidant effect of EEPR by the reducing power of iron has as mechanism to the donation of a hydrogen atom to break the free radical chain through the conversion of Fe^{3+} to Fe^{2+}. (Cushnie and Lamb, 2005; Oyaizu, 1986). Considering the results showed in Table 1, EEPR transformed Fe^{+3} to Fe^{+2}, demonstrating a reducing potential of *P. rigida* and confirmed the response observed by DPPH method.

On the other hand, the lipid peroxidation assay with the β-carotene/linoleic acid co-oxidation system is an *in vitro* test that reproduces physiological situations of oxidative stress, which can lead to cell death in extreme cases by compounds that act on lipid environment (Alam et al., 2013; Stutz et al., 2015). Our data revealed that EEPR had an antioxidant potential against lipid peroxidation by inhibiting oxidative process and was more effective than rutin and quercetin. The lipidic environment favors the action of compounds belonging to terpenoids and steroids that were detected in the phytochemical screening of EEPR.

The acute toxicity test on mice showed that the EEPR was no toxic for the animals. The signs and symptoms of toxicity were also not revealed demonstrating an important finding to the traditional use of *P. rigida*, since the population has used this plant for various medicinal purposes.

The application of acetic acid intraperitoneally produces a painful response characterized by writhe and body stretching. Acetic acid induces the opening of ion channels and transient receptor potential vanilloid 1 (TRPV1) in nociceptive afferent neurons (Ikeda et al., 2001; Julius and Basbaum, 2001). From the viewpoint of inflammatory metabolites, acetic acid promotes the release of TNF-α, interleukin 1β and interleukin 8, prostanoids and bradykinin (Ribeiro et al., 2000). Our results showed a dose-related antinociceptive activity of EEPR (Figure 2), which could be related to inhibition of peripheral mediators, such as prostaglandins. Moreover, this effect may be related to the synergism of different compounds, such as flavonoids, identified in *P. rigida*.

According to Figure 3, EEPR produced significant inhibition in the first and second phases. As clinical pain model, this assay is characterized by a local tissue injury

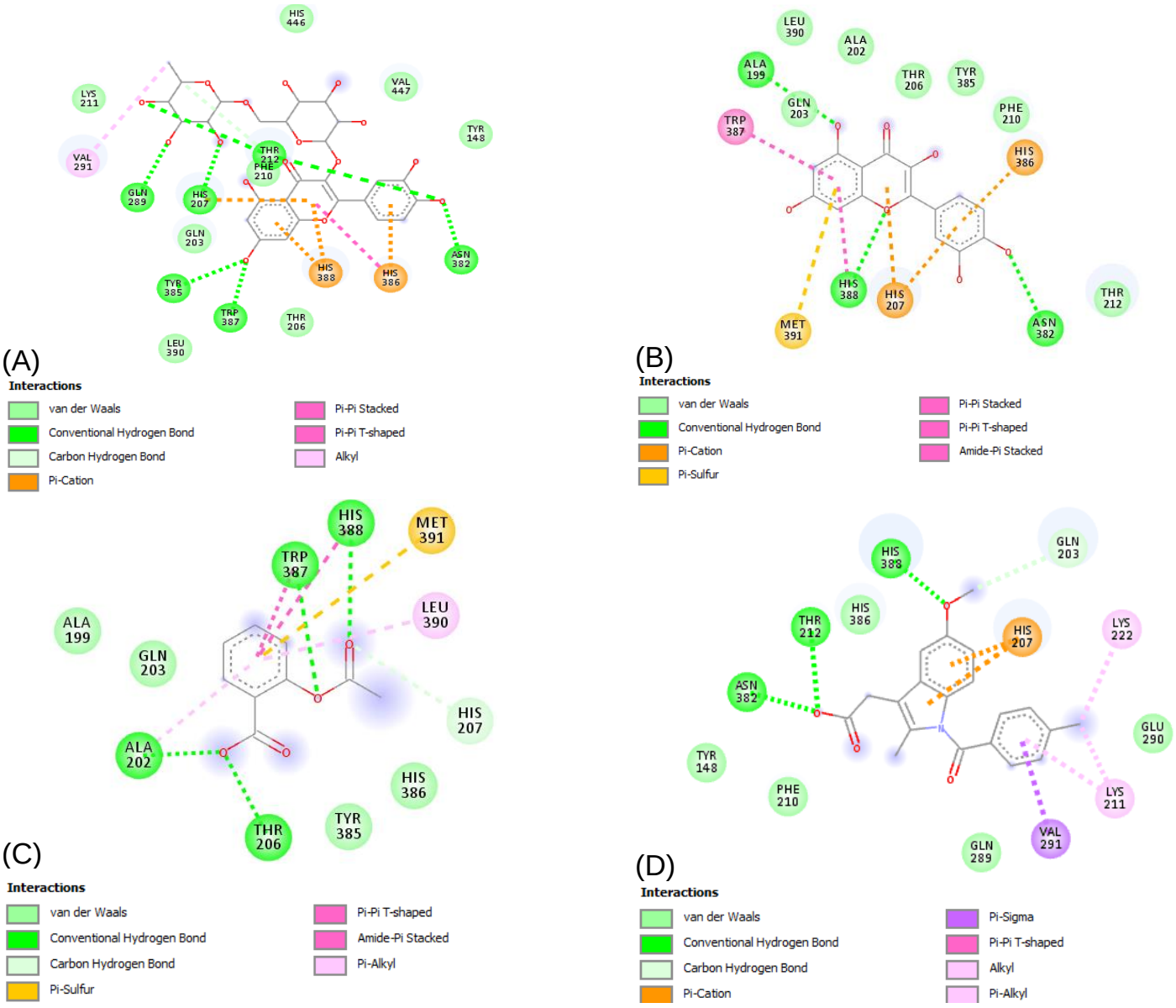

Figure 5. Molecular interactions between ligands and cyclooxygenase-1, showing amino acid binding site. (A) Rutin; (B) Quercetin; (C) Acetylsalicylic acid; (D) Indomethacin.

distinguishing two phases of pain (neurogenic and inflammatory). The neurogenic phase (0-5 min after injection of formalin) causes a direct stimulation of nociceptors, while the inflammatory phase (15-30 min after injection of formalin) is due to the generation of inflammatory mediators (Hunskaar and Hole, 1987). Opioids, as morphine, are able to inhibit both phases, and anti-inflammatory agents (aspirin, indomethacin and dexamethasone) only block the second phase (Hunskaar and Hole, 1987; Le Bars et al., 2001). In this assay, substance P and bradykinin are generated in the neurogenic phase, while other chemical mediators, such as histamine, serotonin and prostaglandin, are involved in the nociceptive response of the second stage (Martins et al., 2006). Considering our results, EEPR inhibited both phases, but rutin and quercetin were active only in the second stage. In this sense, EEPR possess compounds that may exert a central action similar to morphine and peripheral action by the inhibition of inflammation mediators. In addition, rutin and quercetin showed peripheral effect only.

The antinociceptive effect mediated by central mechanisms can be evaluated by the hot plate model. In this model, the thermal stimulus actives TRPV1 and induces paw licking and jumping (Carter, 1991). Once activated, TRPV1 promotes Ca^{2+} influx, actives voltage-dependent Na^{+} channel, depolarizes nociceptive sensory fibers, and propagates the action potential (Julius and

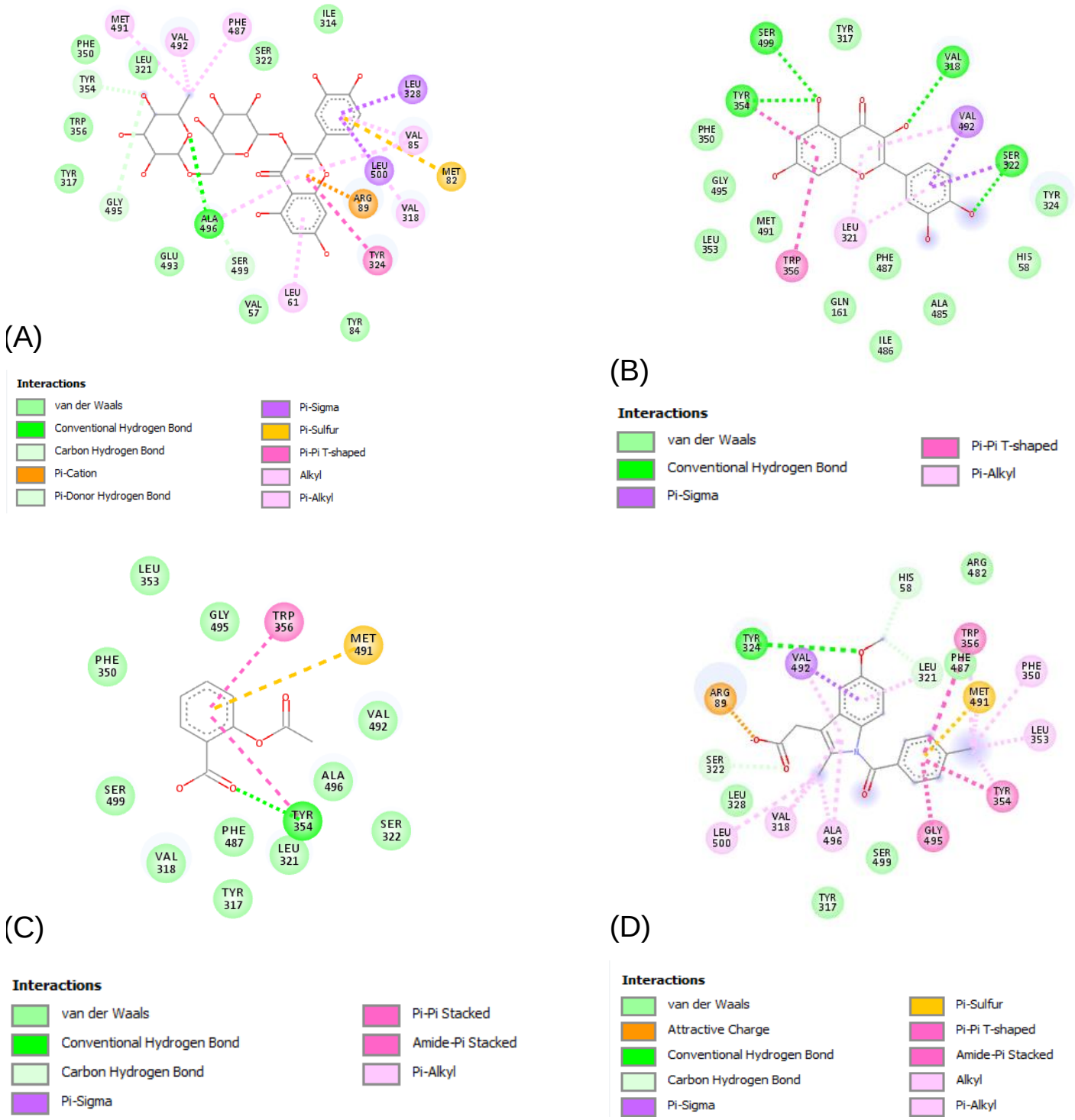

Figure 6. Molecular interactions between ligands and cyclooxygenase-2, showing amino acid binding site. (A) Rutin; (B) Quercetin; (C) Acetylsalicylic acid; (D) Indomethacin.

Basbaum, 2001). According to the presented data (Table 2), EEPR promoted a central effect that confirms the results of the neurogenic phase, and suggests at least a supraspinal modulatory effect. Furthermore, anti-nociceptive action induced by EEPR was not exclusively opioid-dependent system, because naloxone did not totally block this action (Table 2). In this experiment, rutin and quercetin did not contribute with the central effect.

Considering the formalin test, the anti-inflammatory activity of EEPR, rutin and quercetin was corroborated

using the carrageenan-induced paw edema model. This model of acute inflammation is most commonly used to test compounds with anti-inflammatory potential, and involves different phases and inflammatory markers (Winter et al., 1962; Fereidonia et al., 2001). After carrageenan application, serotonin and histamine are mediators found in the first phase (1 to 2 h); kinins are present in the middle phase; the generation of prostaglandins occurs in the late phase (3 to 5 h) (Fereidonia et al., 2001). According to this report, our results showed that EEPR (400 mg/kg) was active on the first phase (2 h) indicating an inhibition of early mediators (as histamine and serotonin), while the effect of EEPR found in the second phase may be justified by a reduction of prostaglandins. Therefore, the present results indicate that EEPR, rutin, quercetin and indomethacin are able to protect the body against acute inflammation.

The anti-inflammatory effect was also confirmed using carrageenan-induced pleurisy model in rats. This model is able to quantify the pleural exudate and inflammatory cells related to inflammation. Anti-inflammatory agents, as indomethacin, reduce the volume of exudate and leukocytes migration between 3 and 6 h after induction of pleurisy (Vinegar et al., 1973; Mikami and Miyasaka, 1982). The tests performed with EEPR, rutin and quercetin in the pleurisy model showed that such products behave as inhibitors of leukocyte migration and pleural exudates formation when given orally.

To test the hypothesis of a possible action mechanism of quercetin derivatives, we evaluated the molecular interaction profile by docking tools. Our results showed that the ligands (rutin and quercetin) are capable of complexing with the inflammatory enzymes (cyclooxygenase-1 and cyclooxygenase-2) more efficient than reference substances (acetylsalicylic acid and indomethacin), since they produced lower free energy between the molecular interactions observed by affinity binding (Table 3 and Figures 5 and 6). These findings may corroborate the anti-inflammatory action of EEPR observed in the *in vivo* tests and they are in agreement with literature data (Pany et al., 2013).

In summary, the present study showed that EEPR possesses antioxidant, antinociceptive and anti-inflammatory effects that must involve peripheral and central components and could be associated with the synergism of substances found in *P. rigida*. In addition, rutin and quercetin derivative found in EEPR appear to contribute for these effects, possibly through at least an inhibitory action on signalling pathways of inflammation mediators. The results also corroborate the medicinal uses of *P. rigida*, but new scientific evidences are necessary for a better knowledge of their therapeutic applications.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

This study was supported by FAPEMIG, CAPES, CNPq, and UFJF. The authors are grateful to Éder Luis Tostes and Jésus de Paula Sarmento, for the technical support.

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Vol. 11(10), pp. 207-217, 10 March, 2017
DOI: 10.5897/JMPR2016.6205
Article Number: FA395E563206
ISSN 1996-0875

http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

In vitro cytotoxic potential of Yacon (*Smallanthus sonchifolius*) against HT-29, MCF-7 and HDFn cell lines

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Received 27 July, 2016; Accepted 6 March, 2017

Yacon (*Smallanthus sonchifolius*) tubers and leaves have been used widely as foodstuff and as remedy for urinary ailments, muscle pain, hyperlipidemia and diabetes mellitus. Recent studies have investigated on isolating active components for their anti-cancer potential against melanoma, cervical cancer and colon cancer. In this study, the cytotoxicity potential of hexane, methanol and DCM extracts of yacon leaves was assessed against MCF-7 (breast cancer), HT-29 (colon cancer) and HDFn (normal human dermal fibroblast) cell lines by using AlamarBlue® assay. Results showed significant reduction in cellular viability of MCF-7 cell lines caused by hexane, methanol and DCM extracts in a dose dependent manner, with DCM being the most potent. The DCM extract also produced significant cytotoxic activity against HT-29 cells, with IC_{50} lower than 5-fluorouracil. Effect on HDFn showed that three yacon extracts produced significantly lower cytotoxicity compared to drug controls with the DCM extract showing the least toxicity.

Key words: Yacon, alamar, breast, colon, cancer, MCF-7, HT-29.

INTRODUCTION

Cancer is one of the leading causes of mortality not only in the Philippines but worldwide, with an incidence rate that is observed to be linearly increasing through time (Cancer Research UK, 2016). Numerous studies have investigated on every aspect of malignancy, including types, causes, clinical presentation, pathologic basis, genetics, prognosis, diagnosis and treatment. These scientific inquiries have led to significant improvements on cancer management. However, the modern era is still facing the constant dilemma of treatment toxicity. Yacon (*Smallanthus sonchifolius* Poepp. & Endl.) is a perennial plant that forms underground tuberous roots. This

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member of the sunflower family (Asteraceae) is a native herb found in the Andean regions, and is currently being cultivated in the Mountain Province. Fresh yacon tubers are edible, yellowish white, crisp and juicy similar to apple or sinkamas, with sweetness that increases with storage. The root crops are usually eaten raw, but can also be prepared into syrups, jams and other foodstuff (Graefe et al., 2004). Aside from household consumption as food, there are a number of ethnomedical uses for yacon. The tubers were eaten raw in South America as diuretic for urinary ailments. Similarly, in Bolivia, decoctions of the leaves were used as home remedy for cystitis, kidney and even liver problems. Peruvians alternatively prepare leaves into a warm poultice for treatment of muscle and joint pains (Graefe et al., 2004). In Brazil, leaves of yacon were taken in the form of tea for control of diabetes (Genta et al., 2009).

Yacon tubers consist mainly of fructans with a structure that is of the inulin type, that is, β(2→1) fructofuranosylsaccharose (Ojansivu et al., 2011). This content makes yacon tubers marketable as sucrose substitutes and are considered dietetic. Additionally, fructans have favorable influence on the human intestinal flora and can modify certain types of lipid disorders. Since humans have no enzyme capable of hydrolyzing the β(2→1) bond, these fructans also serve as dietary fiber (Ojansivu et al., 2011). Recently, oligofructans have been classified as prebiotics (Pedreschi et al., 2003). These compounds are transported to the colon and fermented by selected species of gut micro-flora, especially *Bifidobacterium* and *Lactobacill*us, both indicators of a balanced gut flora. The prebiotic effect of yacon tuber extracts has been demonstrated by their fermentation by these gut bacteria, *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium bifidum* (Valentova and Ulrichová, 2003). Studies have shown that prebiotic consumption favorably modifies gut flora composition and its metabolic activities. Perhaps in a similar manner, yacon tuber consumption also modulates lipid metabolism, calcium absorption and immune response. β(2→1) fructans are related to β-glucans, native polysaccharides found in yeast and fungi, serving as non-specific immunostimulators (Valentová et al., 2003). They bind to macrophages, activate them and initiate the immunity cascade. β-glucans are recommended for the treatment of immunity defects, infections, allergies, chronic fatigue syndrome, high cholesterol levels, stomach problems and as an adjuvant in carcinoma therapy. Yacon tubers are also rich in free fructose, glucose and sucrose (Valentova et al., 2006).

The fructooligosaccharides (FOS) extracted from yacon roots were also found to have hypolipidemic effects on diabetic rats. A significant decrease in fasting plasma triacylglycerol and very low-density lipoprotein levels were observed, along with increased insulin-positive pancreatic cell mass distributed in small cell clusters within the exocrine parenchyma (Habib et al., 2011). The positive metabolic effects of yacon root extracts were further tested in diabetes. Aqueous extracts were effective in controlling water and food consumption, hyperglycemia and dyslipidemia, and promote the reduction of liver enzymes, suggesting a hepatoprotective effect in rats with drug-induced diabetes mellitus type 1 (Ornelas et al., 2013). FOS extracted from yacon roots was also found to have preventive effect against *Salmonella typhimurium* enteric infection. When given orally up to 30 days, FOS from yacon enhanced non-specific immunity, such as increasing the total IgA, which improves the immunological intestinal barrier, thereby preventing pathologic colonization by *S. typhimurium* (Velez et al., 2013). The high concentration of fructans in yacon roots was also discovered to have potential for colon cancer prevention. A significant reduction in number and multiplicity of aberrant crypt foci and in number of invasive adenocarcinomas was observed in the groups orally treated with 1% yacon and its symbiotic formulation (yacon plus *L. casei*) (de Moura et al., 2012). Extracts of yacon were also shown to inhibit progression of acute pancreatitis. The inhibitory effect of 1% of yacon extract on dibutyltin dichloride-induced pancreatitis in rats was interpreted based on decreased levels of inflammatory mediators, such as tumor growth factor and cyclooxygenase-2, in yacon-treated subjects (Choi et al., 2012).

Yacon leaves were also extensively studied for physiologic effects on animals. A review on the characteristics of yacon as a functional food (Delgado et al., 2013) states that yacon leaves contain several phenolic compounds that enhance growth of intestinal bacteria with good metabolic properties, inhibiting the attack of pathogens. Hydro-ethanolic crude extracts (400 mg/kg) of yacon leaves given orally to diabetic Wistar rats for 3, 7, 10 and 14 days were shown to significantly decrease fasting and post-prandial serum glucose (Baroni et al., 2008). This finding was further confirmed by another study that utilized methanol, butanol and chloroform extracts, given to Wistar rats at 50, 10 and 20 mg/kg body weight for eight weeks (Genta et al., 2010). This study measured for oral glucose tolerance test and serum insulin, aside from fasting and post-prandial blood glucose. Results showed effective hypoglycemic activity and increased insulin levels. Another study utilized normoglycemic mice and concluded that 100 mg/kg oral dose of yacon leaf tea extract and ent-kaurenoic fraction were both effective in lowering blood glucose levels (Raga et al., 2010). The methanolic extract of yacon leaves yielded ent-kaurenoic acid and related diterpenoid substances. Recently, e*nt*-kaurenoic acid from yacon was found to possess significant antibacterial and antifungal activities (Padla et al., 2012). Extracts of leaves were also found to have *in vivo* radical scavenging activity. Peroxidation of lipids was significantly inhibited, protecting the liver of rats against oxidative injury (Valentova et al., 2003).

Sesquiterpene lactones, namely, enhydrin, uvedalin and sonchifolin, were also isolated from the leaves of yacon (Siriwan et al., 2011). Sesquiterpene lactones are plant products extensively studied for their wide array of biological activities, such as anti-inflammatory, neurocytotoxic and anticancer potentials (Cho et al., 2004). The ones isolated from yacon leaves, specifically enhydrin and uvedalin, are demonstrated to have potent anticancer activity against cervical cancer cell line, specifically by inducing apoptosis-mediated proliferation inhibition via caspase and deactivation of NF-κB (Siriwan et al., 2011). Another study have also shown chemopreventive properties of the sesquiterpene lactones isolated from yacon leaves, with enhydrin, uvedalin and sonchifolin showing stronger chemopreventive activity than parthenolide (Siriwan et al., 2011). The latter is a reference sesquiterpene lactone that has been proven to possess potent chemopreventive properties and is now included in cancer clinical trials (Ghantous et al., 2010). A study exploring on trypanocidal activity of sesquiterpene lactones isolated from yacon revealed that enhydrin, uvedalin and polymatin B efficiently inhibited both the epimastigote and the replicative intracellular amastigotes of *Trypanosoma cruzi* (Frank et al., 2013).

Yacon has also been investigated on its action against colon cancer and melanoma. Scientists used 1,2-dimethylhydrazine to induce colon carcinogenesis in male Wistar rats. Those administered with dried extract of yacon root and a mixture of yacon with a probiotic showed significant reduction in number and multiplicity of aberrant crypt foci and decreased number of invasive adenocarcinomas (De Moura et al., 2012). Another study investigated the anti-oxidant and anti-cancer activities of different organic solvent fractions of yacon root. Hexane fractions showed high growth inhibitory activities against cancer cells (Min et al., 2012). Another study explored the potential of yacon for melanin synthesis inhibition. Yacon leaf extracts exhibited significant anti-melanogenic activity to suppress melanin synthesis in mouse B16 melanoma cells (Ishikawa et al., 2010).

This study aims to establish reliable data on the anticancer activity of yacon extracts, specifically against breast and colon cancer cell lines. Future scientific ventures on acute toxicity, subacute toxicity and human clinical investigations on yacon will greatly benefit from the output of this study. The information generated from this research can also be used in further identification of active components, which will eventually aid in the discovery and synthesis of a novel, plant-derived drug with superior cytotoxic activity and acceptable side effect profile.

MATERIALS AND METHODS

Collection of plant

Yacon leaves were collected from a farm in Misamis Oriental under the management of Doalnara Multi-Purpose Cooperative. Samples of the leaves were sent to the Bureau of Plant Industry for taxonomic identification. The leaves were cleaned and shade dried for more than 4 weeks in average ambient temperature of 32°C and humidity of 64%. The dried materials were ground into powder using a blender and stored in airtight plastic containers and labeled accordingly.

Preparation of plant extract

The finely ground leaves of *S. sonchifolius* (278.62 g) was exhaustively extracted for six consecutive days (two days for each type of solvent) with solvents in increasing polarity starting with hexane, followed by dichloromethane, and lastly with methanol. For every extraction, the collected crude extracts were concentrated *in vacuo* using a Buchi rotavapor at a maintained temperature of 45°C. Each extraction afforded three crude extracts labeled as SsH for the hexane extract, SsD for the dichloromethane extract, and SsM for the methanol extract. Small amounts of each crude extract (0.2029 g for SsM, 0.6832 g for SsD, and 0.3888 for SsH) were prepared into 100 µg/ml using 0.2% dimethyl sulfoxide (DMSO) in complete Dulbecco's modified eagle medium (DMEM) as solvent. This working concentration was then serially diluted (two-fold) to 50, 25, 12.5, 6.25, 3.125, 1.563 and 0.781 µg/ml during treatment on the different cell lines.

Cell culture

Three cell lines were used for this study, namely, breast cancer (MCF-7), colon cancer (HT-29) and normal human neonatal dermal fibroblast (HDFn) cells. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) and 1% antibiotic antimycotic (Invitrogen, USA) in tissue culture flasks (Falcon, USA) and incubated at 37°C, 5% CO_2 and 95% relative humidity. Cell counts were obtained by the trypan blue exclusion method to calculate cell densities to a final of 1×10^4 viable cells per ml. Experiments were performed in flat bottom 96-well microplates (Falcon, USA) seeded with cell densities of 1×10^3 cells per well. The cells were incubated for 24 h before the drug or plant extracts were added. Untreated cells served as negative controls while 0.2% DMSO in complete DMEM as negative vehicle control. After treatment, the cells were incubated for 48 h prior to analysis with the AlamarBlue® assay (Sankara et al., 2013).

AlamarBlue® assay

Ten microliters of AlamarBlue® was aseptically added to each well. The plates were shaken carefully to thoroughly mix the contents. These were then further incubated at 37°C, 5% CO_2 and 95% relative humidity for 4 h. Viable cells in culture reduce blue resazurin in AlamarBlue® into red resorufin, which has maximum absorbance measured at 570 nm using a microplate reader (ELx800, Biotek, USA) (Fotakis et al., 2006; Sankara et al., 2013).

Methotrexate, colchicine, tamoxifen and 5-fluorouracil were used as standard drug controls. Concentrations of these drugs were prepared similarly as that of the extracts using complete DMEM as solvent. The assay was done in triplicates. The percentage of inhibited growth was computed as: 100 – [(absorbance of treated cells/absorbance of untreated cells) × 100].

Statistical analysis

The data were expressed as mean optical density ± standard

Table 1. Mean optical density (AU) from MCF-7 at different concentrations (µg/ml) of *Smallanthus sonchifolius* leaves extracts and controls.

Controls/Plant extracts	Optical density (AU)* at different concentration (µg/ml)							
	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00
C	0.93±0.01	0.92±0.02	0.77±0.03	0.67±0.07	0.45±0.02	0.33±0.02	0.23±0.02	0.15±0.00
FC	0.87±0.02	0.73±0.02	0.55±0.03	0.55±0.04	0.48±0.02	0.37±0.02	0.36±0.02	0.26±0.01
M	1.18±0.48	0.76±0.05	0.72±0.02	0.66±0.04	0.55±0.03	0.47±0.00	0.37±0.00	0.27±0.00
T	1.07±0.39	0.95±0.26	0.80±0.07	0.74±0.01	0.72±0.02	0.70±0.03	0.48±0.01	0.46±0.02
SSH	1.22±0.22	0.96±0.03	0.77±0.02	0.66±0.00	0.44±0.02	0.44±0.04	0.35±0.05	0.24±0.04
SSM	0.96±0.03	0.86±0.01	0.70±0.05	0.66±0.03	0.47±0.02	0.47±0.01	0.36±0.00	0.26±0.01
SSD	0.88±0.01	0.76±0.03	0.66±0.03	0.55±0.02	0.44±0.03	0.37±0.01	0.24±0.04	0.27±0.00
DMSO	0.92±0.23	0.74±0.02	0.72±0.03	0.70±0.02	0.70±0.02	0.68±0.03	0.67±0.03	0.66±0.04

*Mean ± SD, n=3. C: Colchicine; FC: 5-fluorouracil; M: methotrexate; T: tamoxifen; SSH: hexane extract; SSM: methanol extract; SSD: dichloromethane extract; DMSO: solvent.

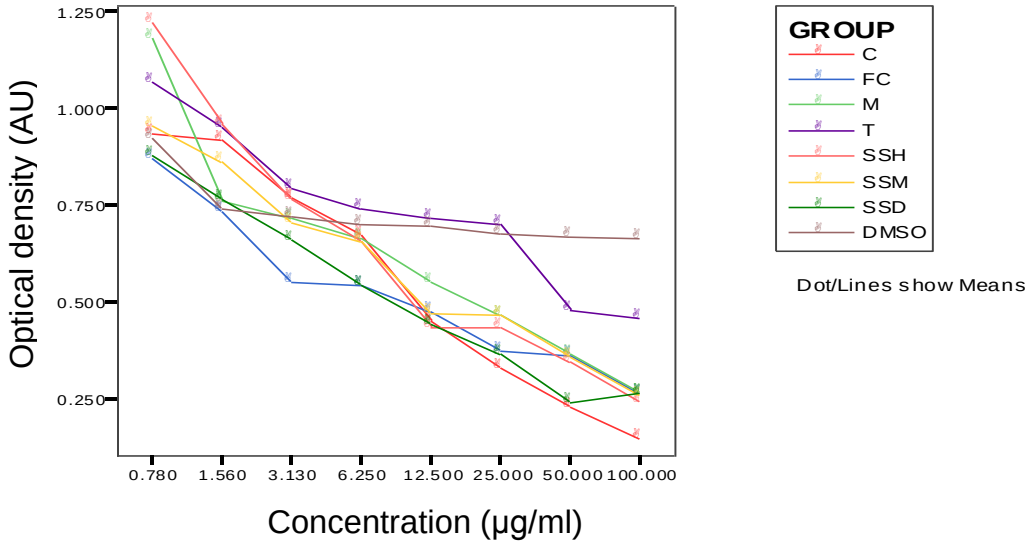

Figure 1. Mean optical density (AU) vs. concentration (µg/ml) – MCF-7.

deviation (SD). Analysis of variance (ANOVA) was used to assess significant differences between controls and plant extracts. IC_{50} for extracts and controls were computed from the generated dose-response curves.

RESULTS

MCF-7 Cell Line

The next set of tables and figures shows the cytotoxicity effect of increasing concentrations of yacon leaves extracts and controls on breast cancer cells. The measured optical densities are tabulated in Table 1, and plotted against concentration in Figure 1. The computed percentages of cell viability inhibition are shown in Table 2 and Figure 2. There is an observed linear decrease in optical density and increase in cellular growth inhibition with increasing concentration of the three extracts. ANOVA analysis revealed significant difference (p value < 0.001) from negative control for the three extracts at concentrations 12.5, 25 (except for hexane extract), 50 and 100 µg/ml, and no significant difference from the positive controls at all concentration levels, except for tamoxifen.

The IC_{50} were computed using log-linear regression dose-response curve and are shown in Table 3, along with measure of linearity (r^2), slope and their respective confidence intervals. The three extracts significantly reduced viability of cells in dose-dependent manner, with the DCM extract being the most potent. The IC_{50} for the hexane and methanol extracts were 32.08 and 37.44 µg/ml, respectively. These values are higher than the

Table 2. Percentage of inhibited MCF-7 at different concentrations (µg/ml) of *Smallanthus sonchifolius* leaves extracts and controls.

Controls/Plant extracts	% Cell Inhibition at Different Concentration (µg/ml)*							
	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00
C	-22.09±1.64	-17.74±2.63	3.19±4.28	15.71±8.84	40.99±2.74	56.57±2.39	69.85±2.55	80.46±0.33
FC	-13.68±3.01	6.07±2.88	30.48±3.88	31.58±5.43	37.64±2.08	50.98±1.98	52.46±2.57	65.75±0.59
M	-54.51±63.06	2.09±6.37	9.70±2.46	16.79±4.56	28.07±4.18	38.88±0.38	51.98±0.15	65.05±0.13
T	-39.87±50.30	-22.05±32.89	-.13±8.40	6.85±1.52	6.14±2.14	7.99±3.47	37.21±1.77	39.55±2.28
SSH	-59.91±28.12	-23.03±3.74	3.27±2.36	17.67±0.13	43.08±2.42	42.77±5.60	54.64±6.10	68.29±5.53
SSM	-25.05±3.36	-10.60±1.04	11.29±6.41	17.46±4.27	38.34±2.11	38.93±0.91	52.90±0.20	66.32±0.83
SSD	-15.12±1.17	1.97±4.17	16.75±3.84	31.62±2.82	42.34±4.39	51.90±0.60	68.28±4.67	65.09±0.55
DMSO	-20.74±30.61	4.91±2.88	9.15±3.72	12.16±2.68	8.83±2.54	11.10±4.17	12.33±4.14	12.75±4.95

*Mean±SD, n=3. C: Colchicine; FC: 5-fluorouracil; M: methotrexate; T: tamoxifen; SSH: hexane extract; SSM: methanol extract; SSD: dichloromethane extract; DMSO: solvent.

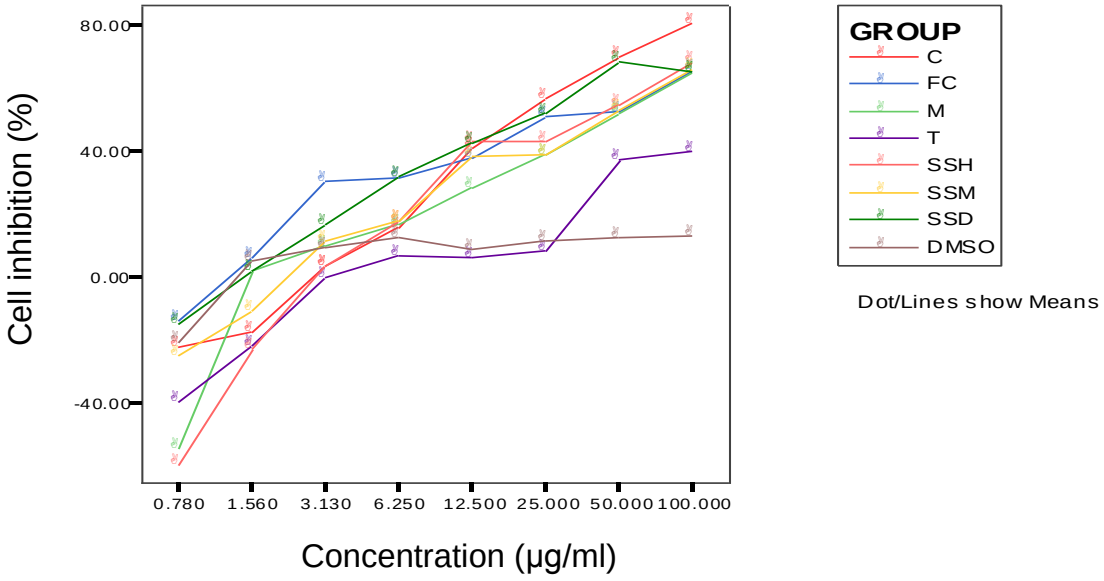

Figure 2. percentage cell inhibition vs. concentration (µg/ml) – MCF-7.

Table 3. IC_{50} values and other dose-response curve parameters against MCF-7 for *Smallanthus sonchifolius* leaves extracts and controls.

Controls/Plant extracts	r^2	Intercept	95%CI		Slope (coefficient for dose)	95%CI		IC_{50}
			Lower	Upper		Lower	Upper	(µg/ml)
C	0.98	-21.81	-25.85	-17.78	23.03	21.53	24.53	22.61
FC	0.92	0.65	-4.67	5.96	14.69	12.72	16.66	28.77
M	0.69	-24.59	-40.96	-8.22	20.35	14.28	26.42	39.06
T	0.61	-28.31	-42.65	-13.98	15.04	9.72	20.36	182.42
SSH	0.89	-35.15	-45.51	-24.78	24.55	20.71	28.39	32.08
SSM	0.97	-16.00	-19.87	-12.12	18.22	16.78	19.65	37.44
SSD	0.96	-4.91	-9.15	-0.66	17.33	15.76	18.90	23.77
DMSO	0.20	-3.94	-12.84	4.95	4.71	1.41	8.01	95092.08

C: Colchicine; FC: 5-fluorouracil; M: methotrexate; T: tamoxifen; SSH: hexane extract; SSM: methanol extract; SSD: dichloromethane extract; DMSO: solvent.

Table 4. Mean optical density (AU) from HT-29 at different concentrations (µg/ml) of *Smallanthus sonchifolius* leaves extracts and controls.

Controls/Plant extracts	Optical density (AU)* at different concentration (µg/ml)							
	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00
C	0.88±0.02	0.76±0.00	0.65±0.01	0.55±0.00	0.45±0.02	0.33±0.02	0.27±0.01	0.17±0.01
FC	0.86±0.01	0.76±0.00	0.66±0.01	0.55±0.01	0.45±0.02	0.34±0.02	0.25±0.01	0.13±0.01
M	0.87±0.01	0.78±0.02	0.74±0.02	0.65±0.03	0.63±0.02	0.47±0.01	0.43±0.02	0.24±0.01
T	0.84±0.03	0.75±0.02	0.73±0.03	0.65±0.00	0.56±0.01	0.55±0.02	0.55±0.02	0.52±0.02
SSH	0.84±0.03	0.75±0.01	0.75±0.01	0.64±0.01	0.53±0.01	0.52±0.01	0.52±0.02	0.50±0.01
SSM	0.85±0.00	0.74±0.01	0.73±0.01	0.63±0.01	0.53±0.01	0.52±0.01	0.52±0.01	0.51±0.01
SSD	0.86±0.01	0.65±0.03	0.54±0.02	0.55±0.03	0.44±0.02	0.33±0.02	0.25±0.01	0.13±0.02
DMSO	0.85±0.03	0.75±0.03	0.73±0.02	0.68±0.01	0.65±0.00	0.65±0.00	0.64±0.02	0.64±0.02

*Mean ± SD, n=3. C: Colchicine; FC: 5-fluorouracil; M: methotrexate; T: tamoxifen; SSH: hexane extract; SSM: methanol extract; SSD: dichloromethane extract; DMSO: solvent.

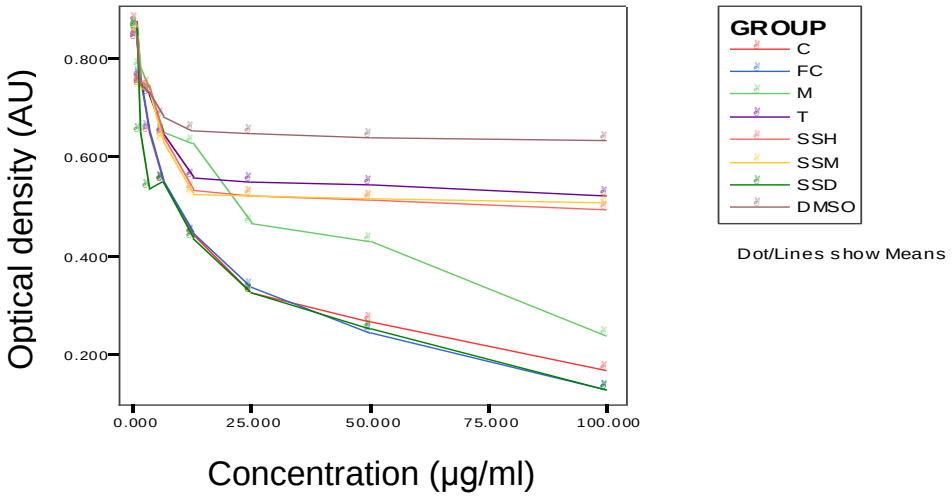

Figure 3. Mean optical density (AU) vs. concentration (µg/ml) – HT-29.

computed IC_{50} for colchicine and 5-fluorouracil, but are significantly lower than the IC_{50} of tamoxifen and methotrexate. The DCM extract has the lowest IC_{50} at 23.77 µg/ml, which is significantly lower than the positive controls, except for colchicine.

HT-29 cell line

Table 4 and Figure 3 show cytotoxic effect of the Yacon leaves extracts and drug controls on colon cancer cells. Decreasing optical densities were observed in a linear fashion after treatment with increasing concentrations of the three Yacon extracts. Percentage cell growth inhibition was computed based on these values, and are shown in Table 5 and plotted against increasing concentrations in Figure 4. The DCM extract significantly reduced cell viability in a dose-dependent manner. ANOVA analysis showed significant difference ($p < 0.001$) of optical density and cell viability from the negative control, and no significant difference from positive controls (5-flurouracil and colchicine) starting from 3.125 µg/ml concentration of DCM extract.

Dose-response curve parameters were generated using log-linear regression to compute for the IC_{50} (Table 6). Hexane and methanol extracts did not exhibit significant cytotoxicity (IC_{50} > 100 µg/ml). On the other hand, the IC_{50} for the DCM extract is 14.32 µg/ml, which is lower than all the positive drug controls, including 5-fluorouracil. This indicates potent cytotoxicity effect produced by the DCM extract.

HDFn cell line

Effect on normal human cell line was assessed by

Table 5. Percentage of inhibited HT-29 at different concentrations (µg/ml) of *Smallanthus sonchifolius* leaves extracts and controls.

Control/Plant extracts	% Cell inhibition at different concentration (µg/ml)*							
	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00
C	1.65±1.92	-0.44±0.43	11.77±0.68	22.81±0.53	46.09±2.75	61.62±1.73	71.68±1.10	79.86±1.61
FC	3.18±0.85	-1.06±0.13	11.37±0.82	22.58±1.54	45.80±1.90	60.33±2.14	74.25±0.88	84.58±0.95
M	2.70±1.17	-3.44±2.89	0.18±2.73	8.74±3.66	23.77±2.46	45.23±0.68	54.67±2.11	71.78±1.52
T	5.92±2.86	0.75±2.06	1.85±3.94	9.16±0.32	32.45±1.47	35.45±2.79	42.60±2.31	38.76±2.42
SSH	5.28±3.70	0.57±0.88	-0.90±1.52	9.91±1.46	35.47±1.38	38.85±1.66	45.89±1.58	42.00±1.29
SSM	4.08±0.47	1.85±1.05	0.72±0.67	11.59±0.99	36.32±1.15	38.62±1.04	45.72±0.90	40.40±1.17
SSD	3.30±0.81	13.77±3.91	27.29±3.17	22.95±3.93	47.34±2.20	61.62±2.36	73.23±1.47	84.66±2.13
DMSO	5.09±3.72	1.15±3.36	0.99±2.33	4.25±1.54	20.82±0.44	23.98±0.49	32.81±1.96	25.64±2.26

*Mean ± SD, n=3. C: Colchicine; FC: 5-fluorouracil; M: methotrexate; T: tamoxifen; SSH: hexane extract; SSM: methanol extract; SSD: dichloromethane extract; DMSO: solvent.

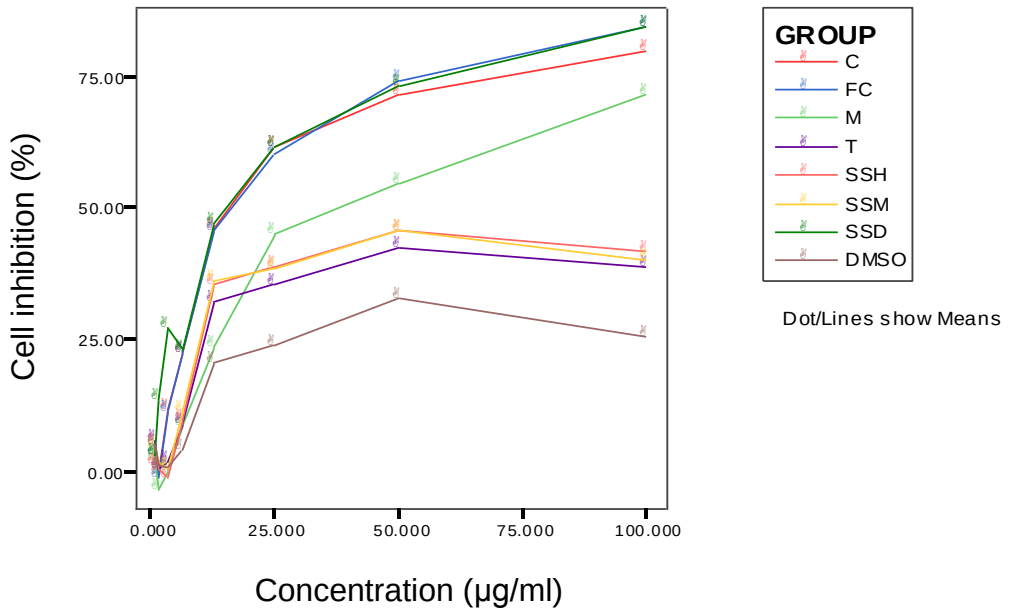

Figure 4. Percentage of cell inhibition vs. concentration (µg/ml) – HT-29.

Table 6. IC_{50} values and other dose-response curve parameters against HT-29 for *Smallanthus sonchifolius* leaves extracts and controls.

Controls/Plant extracts	r^2	Intercept	95%CI		Slope (coefficient for dose)	95%CI		IC_{50}
			Lower	Upper		Lower	Upper	(µg/ml)
C	0.96	-3.57	-7.98	0.834	18.56	16.93	20.2	17.93
FC	0.96	-4.15	-8.88	0.57	19.17	17.42	20.93	16.86
M	0.89	-9.14	-15.69	-2.59	15.87	13.45	18.3	41.53
T	0.82	-0.21	-5.59	5.16	9.67	7.68	11.67	179.89
SSH	0.82	-1.38	-7.4	4.62	10.79	8.56	13.02	116.96
SSM	0.83	-0.49	-6.09	5.11	10.51	8.43	12.59	122.00
SSD	0.96	4.56	0.47	8.65	17.07	15.56	18.59	14.32
DMSO	0.76	-0.17	-4.58	4.25	6.66	5.02	8.3	1868.76

C: Colchicine; FC: 5-fluorouracil; M: methotrexate; T: tamoxifen; SSH: hexane extract; SSM: methanol extract; SSD: dichloromethane extract; DMSO: solvent.

Table 7. Mean optical density (AU) from HDFn at different Concentrations (µg/ml) of *Smallanthus sonchifolius* leaves extracts and controls.

Control/Plant extracts	Optical density (AU)* at different concentration (µg/ml)							
	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00
C	0.56±0.02	0.55±0.01	0.54±0.02	0.50±0.07	0.45±0.10	0.39±0.06	0.30±0.02	0.24±0.03
FC	0.55±0.01	0.52±0.01	0.52±0.01	0.51±0.01	0.43±0.05	0.39±0.01	0.25±0.00	0.14±0.01
M	0.60±0.05	0.55±0.01	0.53±0.01	0.51±0.02	0.46±0.04	0.36±0.02	0.26±0.01	0.15±0.02
T	0.74±0.01	0.72±0.02	0.67±0.03	0.63±0.02	0.61±0.01	0.58±0.01	0.54±0.04	0.50±0.00
SSH	0.97±0.02	0.86±0.01	0.86±0.00	0.76±0.01	0.74±0.01	0.64±0.04	0.61±0.05	0.54±0.01
SSM	0.97±0.01	0.89±0.02	0.85±0.03	0.75±0.02	0.75±0.00	0.65±0.00	0.64±0.02	0.64±0.02
SSD	0.94±0.03	0.85±0.04	0.84±0.03	0.75±0.01	0.75±0.01	0.68±0.02	0.64±0.00	0.62±0.01
DMSO	0.94±0.03	0.92±0.05	0.89±0.02	0.87±0.02	0.84±0.02	0.76±0.00	0.73±0.01	0.72±0.00

*Mean ± SD, n=3. C: Colchicine; FC: 5-fluorouracil; M: methotrexate; T: tamoxifen; SSH: hexane extract; SSM: methanol extract; SSD: dichloromethane extract; DMSO: solvent.

Table 8. Percentage of inhibited HDFn at different concentrations (µg/ml) of *Smallanthus sonchifolius* leaves extracts and controls.

Control/Plant extracts	% Cell inhibition at different concentration (µg/ml)*							
	0.78	1.56	3.13	6.25	12.50	25.00	50.00	100.00
C	42.23±1.61	36.57±1.63	38.89±1.99	44.37±7.26	47.99±11.08	60.11±5.98	66.56±2.09	74.15±3.39
FC	42.78±0.65	39.31±0.66	41.93±0.91	43.48±0.67	50.21±5.99	59.66±0.51	72.87±1.05	85.19±0.65
M	38.14±4.81	36.88±1.53	40.616±0.64	43.15±1.91	46.92±5.03	62.91±1.86	70.99±1.40	84.34±2.28
T	23.16±0.92	17.09±2.10	24.85±3.41	30.49±2.04	29.66±0.58	39.72±0.99	40.38±3.87	46.74±0.22
SSH	-0.38±2.03	-0.04±0.58	3.27±0.491	16.35±0.61	14.62±1.38	3.47±4.00	3.26±5.93	42.79±0.86
SSM	-0.45±1.23	-2.59±2.04	4.13±4.09	17.24±1.68	13.47±0.13	32.54±0.16	29.48±1.67	32.45±1.68
SSD	2.18±2.88	1.51±4.13	5.56±3.77	16.87±1.45	14.43±1.04	31.19±2.09	29.92±0.23	34.46±1.02
DMSO	1.97±2.82	-6.94±5.85	0.24±2.34	4.13±1.89	3.56±1.77	21.01±0.36	19.86±1.16	24.34±0.38

*Mean ± SD, n=3. C: Colchicine; FC: 5-fluorouracil; M: methotrexate; T: tamoxifen; SSH: hexane extract; SSM: methanol extract; SSD: dichloromethane extract; DMSO: solvent.

treating HDFn with the same concentrations of Yacon extracts and controls used for MCF-7 and HT-29 cell lines. Tables 7 and 8 (plotted as Figures 5 and 6, respectively) show the cytotoxic effects of the extracts and controls on the normal cells. Dose-response curve parameters were generated using log-linear regression to compute for the IC50 (Table 9). Results showed significant higher cytotoxicity effect of the drug controls on normal cells compared to the plant extracts. Data suggest that the Yacon extracts are non-cytotoxic to HDFn normal cells (IC_{50} > 100 µg/ml).

DISCUSSION

Cancer treatment almost always includes chemotherapy and/or radiation, and these cytotoxic processes can lead to life threatening conditions such as severe immune deficiency, cardiomyopathy and development of treatment-related malignancy. The search for safer treatment options continues to be an unrelenting challenge for the scientific community. Recent researches have ventured on plant products, uncovering several anti-cancer potentials from different extracts.

Similar to the findings of previous experiments done by De Moura et al. (2012) and Ishikawa et al. (2010), results of this study showed strong potential of the three yacon extracts to be further investigated as cytotoxic agents against breast cancer. The strong cytotoxic activity of DCM extract against colon cancer also warrants further investigation. The IC_{50} for hexane, methanol and DCM extracts against MCF-7 were 32.08, 37.44 and 23.77 µg/ml, respectively. These are acceptable IC_{50} levels against MCF-7 compared to that observed from drug controls (colchicine with 22.61 µg/ml, 5-fluorouracil with 28.77 µg/ml, methotrexate with 39.06 µg/ml and tamoxifen with 182.42 µg/ml). The IC_{50} for hexane, methanol and DCM extracts against HT-29 were 116.96, 122.00 and 14.32 µg/ml, respectively. The IC_{50} observed from hexane and methanol extracts are significantly higher than the drug controls, indicating poor cytotoxic activity for this cancer cell line. However, the low IC_{50} of DCM (14.32 µg/ml) is noteworthy, as it is significantly lower compared to the IC_{50} values from all the drug

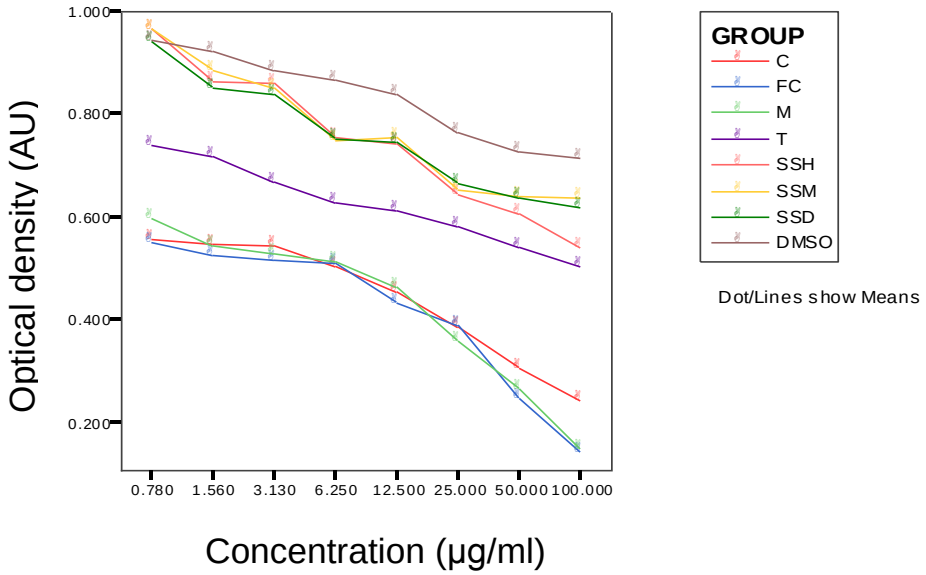

Figure 5. Mean optical density (AU) vs. concentration (µg/ml) – HDFn.

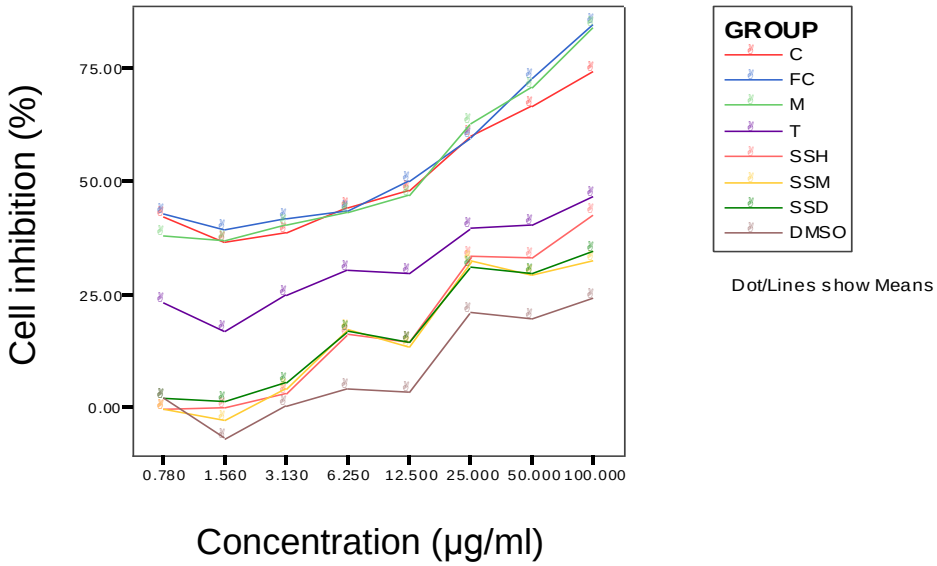

Figure 6. Percentage cell inhibition vs. concentration (µg/ml) – HDFn.

controls (colchicine with 17.93 µg/ml, 5-fluorouracil with 16.86 µg/ml, methotrexate with 41.53 µg/ml and tamoxifen with 179.89 µg/ml).

The three yacon extracts were also observed to be significantly non-cytotoxic to normal HDFn cells. The IC_{50} for colchicine, 5-fluorouracil and methotrexate were 7.38, 5.40 and 6.49 µg/ml, respectively. Tamoxifen produced the highest IC_{50} for the drug controls at 242.19 µg/ml, but this value is still lower than those observed from the plant extracts. The IC_{50} for hexane, methanol and DCM extracts were 252.23, 601.82 and 678.76 µg/ml, respectively. Further investigations should be done on protective effect and mechanism of action of yacon extracts against these cancer cells (Siriwan et al., 2011; Choi et al., 2004).

The DCM extract outstandingly produced lower IC_{50} levels compared to drug controls against MCF-7 (except to colchicine) and HT-29. This extract showed the lowest IC_{50} against HT-29 (14.32 µg/ml), even lower than the IC_{50} observed from the current drug of choice against

Table 9. IC_{50} values and other dose-response curve parameters against HDFn cells for *Smallanthus sonchifolius* leaves extracts and controls.

Control/Plant extracts	r^2	Intercept	95%CI		Slope	95%CI		IC_{50}
			Lower	Upper	(coefficient for dose)	Lower	Upper	(µg/ml)
C	0.77	34.88	29.94	39.82	7.57	5.73	9.40	7.38
FC	0.82	34.81	29.77	39.90	9.01	7.14	10.87	5.40
M	0.86	31.87	27.14	36.61	9.69	7.94	11.45	6.49
T	0.86	19.34	16.69	21.99	5.59	4.60	6.57	242.19
SSH	0.92	-2.94	-6.40	0.52	9.57	8.29	10.86	252.23
SSM	0.88	-1.88	-5.48	1.73	8.11	6.77	9.44	601.82
SSD	0.90	0.46	-2.65	3.57	7.60	6.45	8.75	678.76
DMSO	0.76	-4.66	-8.67	-0.66	6.05	4.56	7.54	8393.45

C: Colchicine; FC: 5-fluorouracil; M: methotrexate; T: tamoxifen; SSH: hexane extract; SSM: methanol extract; SSD: dichloromethane extract; DMSO: solvent.

colon cancer, 5-fluorouracil (16.86 µg/ml).

Conclusion

Conclusively, results of this study feature the potential anti-cancer activity of Yacon extracts, most exceptionally the DCM extract. These extracts showed significant cytotoxic effect against breast (hexane, methanol and DCM extracts) and colon cancer cells (DCM extract), while exhibiting non-cytotoxic activities on the normal human cells compared to existing cytotoxic drugs. Results of this study merit further investigation particularly on the cytotoxic mechanisms of the extracts, which can also be utilized for development of new medicine against cancer.

CONFLICTS OF INTERESTS

The authors have not declared any conflict of interests

ACKNOWLEDGEMENTS

The authors would like to extend their deepest gratitude to Dr. Eleanor Padla (Project Consultant, Department of Microbiology and Parasitology, College of Medicine, DLSHSI), Dr. Jaime Montoya (PCHRD), Dr. Esperanza Cabrera (Center for Natural Sciences and Environmental Research, DLSU), Reinachelle Dacdac (PCHRD Region IV-A), Sigfredo Mata (College of Pharmacy, DLSHSI), Dr. Melchor Frias (Research Division, DLSHSI, PCHRD Region IV-A), Dr. Alicia Catabay (College of Pharmacy), Ning Lacsa Alindogan (College of Pharmacy, DLSHSI), Judy Laudato (Center for Biopharmaceutical Research), Phannie Bautista (Project Staff, Center for Biopharmaceutical Research) and Sirjohn Gorospe (Project Staff, College of Medicine) for their support and assistance. This research is supported by grant from Philippine College of Health Research and Development (PCHRD) of the Department of Science and Technology.

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