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Full Length Research Paper

Ethnobotanical survey of medicinal plants used in malaria management in South Benin

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In Bénin, malaria is a major public health concern, especially for children under five years and pregnant women. Considering the inefficiency of the health centers in some regions, people use plants for their primary health care. This study aimed to identify and document the medicinal plants used in the treatment of malaria in four departments (Littoral, Ouémé, Plateau and Zou) of south Bénin. Data were recorded using interview and field observation. 42 informants including 15 traditional healers and 27 medicinal plants sellers with an average of fourteen years of experience were interviewed. Plants were identified by a botanist from the National Herbarium of Bénin. The study revealed that a total of 34 plants species belonging to 33 genera and 24 families were collected. The most frequently cited plant species was *Chamaecrista rotundifolia*, *Senna siamea* and *Dialium guineense*. Caesalpinioideae and Rubiaceae are the most families mentioned by the informants. The most common preparation method is decoction and the route of administration is oral. The results contributed to the conservation of empirical knowledge of medicinal plants used for the treatment of malaria and could help to identify new research topics in connection with the implementation of Traditional Medicines.

Key words: Medicinal plants, malaria, traditional knowledge, Bénin.

INTRODUCTION

Plants have been used for centuries to treat diseases in native communities. The use of medicinal plants as a source of remedy of illness can be traced back over five millennia (Kabita et al., 2015). Many plants, especially those used by traditional healers have numerous pharmacological activities. About, 85% of world population uses traditional medicine for primary health care and the demand is increasing in developed and developing countries (Balcha et al., 2014). Drugs from medicinal plants have been used worldwide in traditional medicine for the treatment of various diseases. Thus, medicinal plants are the main source of drugs in traditional medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Tiwari et al., 2011).

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Figure 1. Traditional remedies and medicinal plants in a market in southern Benin.

All over the world, people have old tradition in the use of herbs for the treatment of several diseases, including malaria. It is an important parasitic disease which has the potential to affect nearly 37% of the world's population and is responsible for 438000 deaths at 2015 (WHO, 2015). It is the most crucial problem of public health in African sub-Saharan countries where 74% of the population lives in area of strong endemic and 18% in epidemic area (Weniger et al., 2008). According to World Health Organization, a total of 95 countries are endemic of malaria in the world and 45 African countries are concerned by this endemic (WHO, 2014, 2015). In Africa, as in other malaria-endemic regions, inaccessibility of health centers, the inequitable distribution of medical staff and socio-cultural attitudes lead people to use traditional medicines for their primary health care (Ngbolua et al., 2013). New antimalarial drugs are also expensive for the majority of population in most countries where malaria is endemic. The cost of malaria in a region of Burkina Faso is high given the economic income of villagers and the fact that in every family there are several episodes of malaria per year. Thus, malaria is a major public health problem in his mortality, but also in its economic weight at the level of the community and the budgets allocated for health services (Guiguemdé et al., 1997).

In Benin and neighboring countries, many plants with antiparasitic properties are widely used in the traditional medicine system. Many antimalarial investigations have also been done on plants used traditionally to treat infectious diseases with satisfactory results. This was demonstrated by results achieved with numerous studies on species from Benin Pharmacopoeia (Bero et al., 2013; Attioua et al., 2012; Djikpo-Tchibozo et al., 2011). Unfortunately, empirical and traditional knowledge are not registered and disappear over time. It is important to focus research on the identification of traditional plant. This study allows identification and documentation of traditional uses of medicinal plants used in the management of malaria in South Bénin.

METHODOLOGY

Study area

Our study was conducted exclusively in the markets of plants (Figure 1) including plants sellers and traditional healers in four departments: Littoral, Ouémé, Plateau, and Zou (Figure 2), selected for their demographic weight, diversified geographical space, and sanitary status.

These regions are relatively humid agroecological zone with two rainy seasons and means annual rainfall varying from 1100 to 1400 mm for Littoral, Ouémé, Plateau (Yabi and Afouda, 2012) and 900 and 1200 mm for Department of Zou. Mean annual temperatures range from 26 to 28°C and vegetation types are semi-deciduous forests or woodland and savannah woodland (Akoègninou et al., 2006). To adequately cover the study area, the surveys were conducted taking into account the movement of the population and sales areas of medicinal plants. The selected markets for survey are listed in Table 1. In these departments, some segments of the population live in slums which are always full of water even during the dry season. This promotes the development of mosquito larvae and thus the development and persistence of parasite vector mosquitoes of the genus *Plasmodium* that causes malaria. Considering this and the failed management of malaria due to inadequate public health services, the use of medicinal plants has become the leading source of drug for the treatment of the disease.

Selection of informants

Before starting data collection, the objectives of the survey are explained to the managers of the selected markets and informants

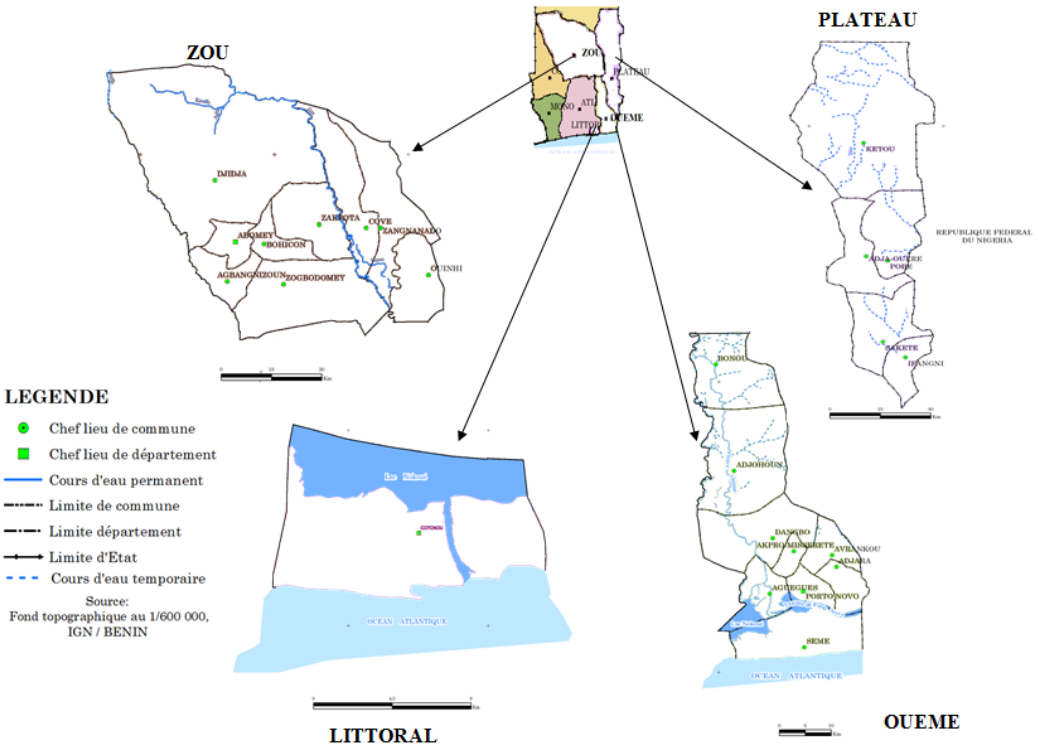

Figure 2. Maps of departments where survey was conducted.

Table 1. Selected markets name and number of plants sellers in study area.

Departments	City	Markets	Number of vendors and traditional healers
Littoral	Cotonou	Kindonou	04
		Mènontin	05
		Vèdoko	03
Ouémé	Porto-Novo	Ouando	04
	Apkro-missérété	Missérété	04
Plateau	Ifangni	Ifangni	03
	Sakété	Takon	02
	Pobè	Ikpinlè	02
		Itadjèbou	03
Zou	Bohicon	Bohicon	07
	Abomey	Houndjro	05

in order to have their consent and facilitate the exchange of information. Indeed, plants and recipes used to treat malaria were recorded and listed as part of the work of the Team of Biochemistry and Bioactive Natural Substances of University of Abomey-Calavi. The final document will be returned to each respondent in order to preserve the data collected and to avoid loss of information through the time. Informants were selected with the support of market managers and resource persons. Forty two (42) informants (29 women and 13 men) with the age over 40 were included.

Data collection

Data were collected from November 2014 to March 2015 in

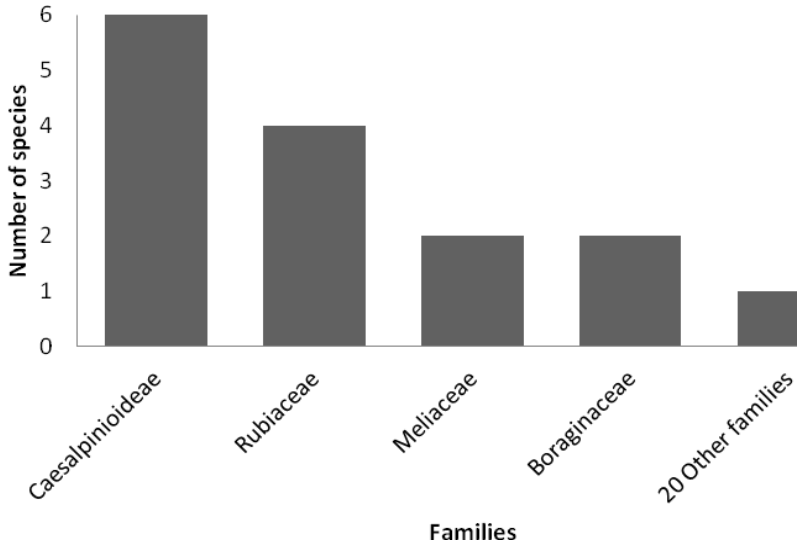

Figure 3. Distribution of reported plants among family.

selected markets. The survey was conducted according to ethnopharmacological approach such as interviews and field visits (Mahomoodally and Muthoorah, 2014). According to informants, questions were asked in local language (Fon, Goun, Yoruba, Nagot or Adja) and data provided are related to the knowledge of malaria (depends on the language), causes and symptoms to diagnose malaria, medicinal plants used in the treatment of malaria, the local name of plants, the parts used, modes of preparation and administration, the availability of plants, the habitats where the species are harvested and the source of the traditional knowledge on medicinal plants.

Plant identification

Collected plants were authenticated by botanist from National Herbarium of University of Abomey-Calavi where voucher numbers were obtained. Many important medicinal plants species are becoming rare and some of them are critically endangered (Qayum et al., 2016). According to the International Union for Conservation of Nature and Natural resource (IUCN), the status of the collected plants has been verified.

Data analysis

Qualitative data were analyzed using the descriptive statistics (percentage) and the results presented in tables and figures. The importance of each plant was calculated based on the relative frequency of citation (RFC) (Tardio and Pardo-De-Santanyana, 2008). The RFC was calculated using formula in which, Fc is number of informants who mentioned the use of the species and N the total number of informants.

RFC = Fc / N

RESULTS

Socio-demographic data of informants

A total of forty-two vendors of plants and healers over 40 years old were interviewed. They include 31 plants sellers comprised 29 women (93.54%), 2 men (6.4%) and 11 healers who are men. The educational level of the informants is variable. Most of them have a low level of formal education. Only nine out of twenty-seven vendors were in primary school while two women and seven men were in secondary school.

Medicinal plants used in malaria management in study area

A total of 34 species used traditionally to manage malaria were recorded in selected markets. These medicinal plants distributed in 33 genera and 24 families were reported to treat malaria by informants in the study area (Table 2). Among reported plants, trees were the most cited (41.18%), followed by shrubs (38.23%) and herbs (20.59%). Among collected species, Caesalpinioideae are the most represented family with six species (17.65%), followed by Rubiaceae with four species (11.76%), Meliaceae and Boraginaceae with two species, respectiveley (5.88%). Other families were represented by one species (Figure 3).

Relative frequency of citation (RFC)

The most frequently cited plant species was *Chamaecrista rotundifolia* with a RFC of 0.88, followed by *Senna siamea*, *Dialium guineense* with RFC of 0.79, and *Dichapetalum madagascariense* with RFC of 0.62. *Dissotis rotundifolia* and *Erhetia cymosa* are also listed among the most used with RFC value of 0.55. Many important medicinal plants species are becoming rare

Table 2. Medicinal plants used to treat malaria in the study area and their IUCN status.

Family	Scientific name	Morphology	Vernacular name	Part use	Method of preparation and administration	Voucher number	RFC	IUCN status
Annonaceae	*Uvaria chamae* P. Beauv.	Shrub	Aylaha, Gbanan (g, f)	Leaves, roots	Decoction, oral	YH241/HNB	0.02	Not yet been assessed
Asteraceae	*Acanthospermum hispidum* DC.	Shrub	Ahouanglon (g, f)	Leaves	Decoction, oral	YH252/HNB	0.26	Not yet been assessed
Bignoniaceae	*Newbouldia laevis* (P. Beauv.) Seem. Ex Bureau	Tree	Désréguéman (g, f)	Leaves	Decoction/maceration, oral	YH254/HNB	0.06	Not yet been assessed
Boraginaceae	*Ehretia cymosa* Thonn. ex Schum.	Tree	Zozoman (g, f)	Leaves	Decoction, oral	YH255/HNB	0.55	Not yet been assessed
	Heliotropium indicum L.	Herb	Kokloviden gbadja (g, f)	Leaves	Decoction, oral	YH256/HNB	0.06	Not yet been assessed
Bromeliaceae	*Ananas comosus* (L.) Merr.	Herb	Ananas (g, f)	Fruit	Decoction, oral	YH258/HNB	0.08	Not yet been assessed
Capparaceae	*Crateva religiosa* DC. Syn.: *C. adansonii* Forst. f.	Tree	Wontonzonzwen (g, f)	Leaves	Decoction, oral	YH260/HNB	0.16	Not yet been assessed
Caricaceae	*Carica papaya* L.	Tree	Kpinman (g, f)	Leaves	Decoction, oral	YH261/HNB	0.08	Not yet been assessed
Caesalpinioideae	*Chamaecrista rotundifolia* (Pers.) Greene	Herb	Aziiman (g, f)	Aerial part	Decoction, oral	YH282/HNB	0.88	Not yet been assessed
	Senna siamea (Lam.) H. S. Irwin & Barneby	Tree	Kassiaman (g, f)	Leaves	Decoction, oral	YH289/HNB	0.79	Not yet been assessed
	Caesalpinia bonduc (L.) Roxb.	Tree	Adjikouindô (g, f)	Root	Infusion, oral	YH280/HNB	0.02	Not yet been assessed
	Caesalpinia pulcherrima (L.) Sw.	Shrub	Hèviviman (g, f)	Leaves, fruit	Decoction, oral	YH281/HNB	0.06	Not yet been assessed
	Dialium guineense Willd.	Tree	Asswènsswèn (g, f); Atitwépa (w); Ewéany (y)	Leaves, bark	Decoction, oral	YH284/HNB	0.79	Not yet been assessed
	Abrus precatorius L.	Shrub	Viviman (g); Amama (y)	Leaves	Maceration, oral	YH302/HNB	0.10	Not yet been assessed
Dichapetalaceae	*Dichapetalum madagascariense* Poir.	Tree	Gbonymisso, Gbaglo (g, f)	Leaves	Decoction, oral	YH267/HNB	0.62	Not yet been assessed
Dracaenaceae	*Sansevieria libertica* Hort. Ex Gerome & Labroy	Herb	Kpoyan (g, f)	Leaves	Decoction, oral	YH233/HNB	0.04	Not yet been assessed
Euphorbiaceae	*Phyllanthus amarus* Schumach. & Thonn.	Tree	Hlènwé (g, f) ; Aribisohou (y)	Leaves	Decoction, oral	YH273/HNB	0.08	Not yet been assessed
Flacourtiaceae	*Flacourtia indica* (Burm.f.) Merr.	Herb	bohoucadjè, Assanhoun (g, f)	Leaves	Decoction, oral	YH274/HNB	0.02	Not yet been assessed
Lamiaceae	*Ocimum americanum* L. Syn.: *O. canum* Sims	Shrub	Hissi-hissi (g, f); Hessi-hessi (a)	Aerial part	Decoction, oral	YH277/HNB	0.08	Not yet been assessed
Malvaceae	*Hibiscus surattensis* L.	Shrub	Kpofin, Kpodèman (g, f)	Leaves	Decoction, oral	YH311/HNB	0.04	Not yet been assessed
Melastomataceae	*Dissotis rotundifolia* (Sm.) Triana	Shrub	Hèhèman (g, f); Ewé eti ékouté (y)	Leaves	Decoction, oral	YH313/HNB	0.55	Not yet been assessed
Meliaceae	*Azadirachta indica* A. Juss.	Tree	Quinininiman (g, f)	Leaves	Decoction, oral	YH314/HNB	0.24	Not yet been assessed
	Khaya senegalensis (Desr.) A. Juss.	Tree	Caïlcédra (g, f)	Leaves	Decoction, oral	YH315/HNB	0.24	Vulnerable A1cd ver 2.3
Moringaceae	*Moringa oleifera Lam.*	Shrub	Kpatiman (g, f)	Leaves	Maceration/decoction, oral	YH318/HNB	0.17	Not yet been assessed
Passifloraceae	*Passiflora foetida* L.	Shrub	Avounyinmitrui (g, f)	Leaves	Decoction, oral	YH322/HNB	0.02	Not yet been assessed
Poaceae	*Cymbopognon citratus* (DC.) Stapf	Herb	Timan/Tchaman (g, f)	Aerial part	Decoction, oral	YH326/HNB	0.04	Not yet been assessed
Rubiaceae	*Gardenia erubescens* Stapf & Hutch.	Tree	Adakplaman (g, f)	Leaves	Decoction, oral	YH331/HNB	0.14	Not yet been assessed
	Morinda lucida Benth.	Tree	Houensi (g, f)	Leaves	Decoction, oral	YH333/HNB	0.17	Not yet been assessed
	Pavetta corymbosa (DC.) F. N. Williams	Shrub	Lohou (g, f)	Leaves	Decoction, oral	YH334/HNB	0.08	Not yet been assessed
	Sarcocephalus latifolius (Sm.) E. A. Bruce	Shrub	Codô (g, f)	Roots	Decoction/infusion, oral	YH335/HNB	0.36	Not yet been assessed
Rutaceae	*Citrus aurantifolia* (Christm. & Panzer) Swingle	Tree	Clessiman (g, f)	Leaves	Decoction, oral	YH337/HNB	0.14	Not yet been assessed

Table 2. Cont'd

Sterculiaceae	*Cola millenii* K. Schum.	Shrub	Alovi aton (g, f)	Leaves	Decoction, oral	YH344/HNB	0.24	Not yet been assessed
Verbenaceae	*Lantana camara* L.	Herb	Hlachiayo (g, f)	Leaves	Decoction, oral	YH350/HNB	0.12	Not yet been assessed
Vitaceae	*Ampelocissus bombycina* (Baker) Planch.	Shrub	Têkplê (g, f)	Leaves	Decoction, oral	YH352/HNB	0.10	Not yet been assessed

f: fon, g: goun, y: yoruba, w: watchi, a: adja

and some of them are critically endangered according to the red list of International Union for Conservation of Nature and Natural resource.

Part used and mode of administration

The survey showed that leaves are the frequently used organs (73.50%), followed by roots and fruits (8.82%), aerial part (5.88%) and bark (2.94%) (Figure 4a). According to collected data, decoction (91.20%) is the most common method of preparation followed by maceration (5.90%) and infusion (2.90%) (Figure 4b). They are mostly administered orally with a dosage ranged between 1 and 3 doses per day. Preparation methods and doses are variable and often random. The only measurable dose is the use of small glass called "T*alokpémi*" corresponding to 40 ml. Regarding the availability of species, vendors have regular suppliers. Sometimes, the plants are domesticated by traditional healers. The healers also purchase plants in the markets.

Causes and symptoms of malaria according to informants

During the survey, all informants mentioned that malaria was caused by mosquito bite called "*Zanssoukpè* or *Gnam-gnamou*" which means "Night Fly" in local language *Fon*, *Goun* and *yoruba* (Table 3). This knowledge of malaria could be explained by the extensive education campaign conducted by the Ministry of Health and the Association of traditional healers. Thirty informants out of forty two reported that working in sun can also cause malaria. This was consistent with the common local name of malaria "*hwessivozon*" which means "sun disease". Consumption of avocado, peanuts or peanut oil were also mentioned (Table 3). The most important symptoms cited by informants were fever/pain (88.10%) and headaches (78.57%) (Table 3). Vomiting (52.38%) and appetite loss (47.62%) were also reported as malaria symptoms.

DISCUSSION

Malaria is the most devastating parasitic disease affecting all developing countries, causing severe emotional and economic disorders and thus delays the progress of nations. The fight against malaria is still difficult because there is no credible vaccine for malaria treatment or drug for which the resistance effect is not reported. To rectify this situation, many recipes of medicinal plants are generally used in the treatment of malaria. The species collected in this study are also involved in the treatment of several diseases in Benin and neighboring countries.

The majority of the species mentioned by the informants belongs to the family of Caesalpinioideae and Rubiaceae. In a survey conducted on plants traditionally used to treat malaria in the maritime region of Togo, it has been shown that Rubiaceae is the most cited family (Koudouvo et al., 2011). In the great diversity of plant species used in the management of malaria, Rubiaceae and Caesalpinioideae are generally the most represented (Adjanohoun et al., 1989; Asase et al., 2010; Kamagaté et al., 2014). This is consistent with our results. Among the recorded species, *C. rotundifolia* (RFC 0.88), *S. siamea* (RFC 0.79) and *D. guineense* (RFC 0.79) were frequently quoted by the informants and highlighted their importance in malaria management. The most cited species *C. rotundifolia* is found in Littoral, Ouémé, Zou Department in Benin (Akoègninou et al., 2006) and in the savannas of Ghana and Nigeria where the aqueous decoction of the whole plant is indicated for the treatment of jaundice and malaria (Adjanohoun et al., 1989). In Benin, decoction of *S. siamea* root, bark and leaves is indicated orally for the treatment of malaria (Adjanohoun et al., 1989). *S. siamea* is also used traditionally in the south-west Nigerian as an antimalarial remedy (Batista et al., 2009). The similarity of results could be justified by proximity between study area in Bénin and Nigeria. The leaves of *D. guin*eense were used alone or in combination with other plants by traditional healers and local populations as antimalarial in Bénin (Adjanohoun et al., 1989). The common vernacular names in the study area were *Assonswen* (Fon and Goun, southern region), *Anwin* (Yoruba, middle and southern region). *D. guin*eense can be found in West African countries

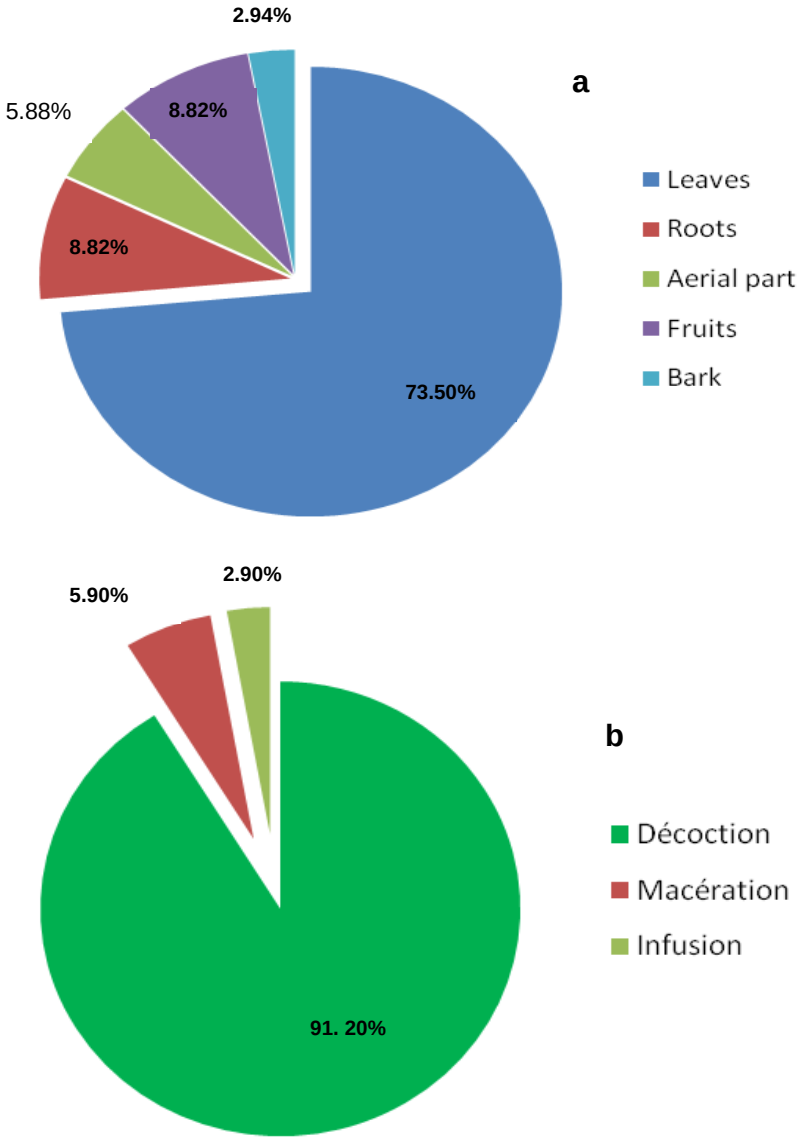

Figure 4. (a) Plants parts used in the management of malaria in the study area; (b) Preparation methods of collected plants in study area.

such as Ghana where it is known as Yoyi, Sierra Leone, Senegal, and Nigeria (Adumanya et al., 2013; Akinpelu et al., 2011; Nwosu, 2004). The stem bark and leaves of the same species are used traditionally to treat malaria fever in Nigeria (Balogun et al., 2013).

Among the recorded species in this study, 8 species were frequently quoted by the informants and highlighted their importance in malaria management. Other traditional uses and *in vitro* antiplasmodial activity of four species with highest citation frequency were also reported by previous studies (Adjobimey et al., 2004; Bero et al., 2009; Erhenhi and Obadoni, 2015; Fah et al., 2013; Kazhila, 2015).

The obtained data indicated that the traditional healers used various parts of the medicinal plants for preparation of antimalarial remedies. However, leaves were the major parts used. Similar reports had been already recorded in several studies (Koudouvo et al., 2011; Asase et al., 2010; Nguta et al., 2010; Balcha, 2014). Contrary to the devastating effect that the use of roots and bark can cause to plant biodiversity, the high frequency of use of leaves in recipes is a great advantage for the preservation of plant biodiversity. Then, the use of leaves is less dangerous than to the use of underground parts (roots, stem, bark), or the use of whole plants (Giday et al., 2003; Zheng and Xing, 2009). However, harvesting the leaves for therapeutic purposes is not without effect on the regeneration of biodiversity because it could limit the vegetative reproduction that leads to the development of flowers, fruits and natural regeneration of wild plants

Table 3. Causes and symptoms of malaria cited by informants.

Causes	Number of informants	Symptoms	Number of informants
Mosquito bite	42	Fever and pain	37
Working in the sun	30	Headache	33
Working or Walking in the rain	9	Vomiting/mouth sores	22
Consumption of oil	11	Nausea	08
Working in the rain	9	Body weakness	04
Avocado consumption	13	Vomiting, pale face	09
Peanut and oil consumption	4	Appetite loss	20
peanut consumption	5	Dizziness, Sweating	10

(Cunningham, 2001).

In this study, it was found that the main method of transmission of traditional knowledge of medicinal plants was parental inheritance. It was also noted that knowledge is transmitted orally. Analogous to this study, traditional knowledge on medicinal plants in most communities is often passed from one generation to another usually via word-of-mouth (Yetein et al., 2013). From this study, we notice that the use of medicinal plants differs from a department to another according to the source of information. This situation testifies the complexity of the medicine domain and the traditional pharmacopeia. People are greatly concerned about the efficacy and side effects of many synthetic drugs. Hence, they choose herbal medicines as a safe and natural alternative treatment for many health problems. The use of medicinal plants is growing, because herbs are always the source of alternative medicine for primary health care.

Conclusion

Our investigation allowed the registration of plants and recipes traditionally used in the treatment of malaria in four departments of southern Benin. These results show the importance of the use of plants in the treatment of human diseases. The information listed was recorded in a document in order to preserve the data collected and to avoid loss of information through the time. The results obtained for our study could also help to identify new research topics in connection with the implementation of traditional medicines.

Conflict of interests

The authors have not declared any conflict of interest.

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Effect of crude extracts from the root of *Stemona tuberosa* Lour. on the replication of *Autographa californica* multiple nucleopolyhedrovirus

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The purpose of this research was to investigate the effect of crude extracts from the root of *Stemona tuberosa* Lour. on the replication of *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV). Cytotoxicity of crude hexane, dichloromethane and ethanol extracts from the root of *S. tuberosa* was tested against *Spodoptera frugiperda* cell line (Sf9) using MTT assay. The cytotoxic effect, represented as CC_{50} (µg/ml) was observed after 48 and 96 h. It was shown that dichloromethane extract was more toxic than hexane and ethanol extracts and 96-h CC_{50} for the dichloromethane extract was 1,708.98 µg/ml. Crude dichloromethane extract at the concentration of 31.25 µg/ml was then tested on *Ac*MNPV. The extract was added after 1 h post-infection of *Ac*MNPV at the multiplicity of infection (MOI) of 2, in Sf9 cell line cultivated *in vitro*. No significant difference between the percentage of infected cells in the control and the test sample with crude dichloromethane extract was found. The average number of polyhedra (OBs/ml) in the control ($5.11\times10^6\pm0.63$ OBs/ml) was not significantly different from the test sample ($4.19\times10^6\pm0.31$ OBs/ml). However, there was significant difference between the average virus titer (budded virus, BV) in the control ($2.06\times10^8\pm0.71$ PFU/ml) and the test samples ($2.65\times10^8\pm0.79$ PFU/ml). Crude dichloromethane extract (31.25 µg/ml) did not toxic to Sf9 cells but it could enhance *Ac*MNPV replication in Sf9 cell line cultivated *in vitro*. It could be concluded that *S. tuberosa* can be a good candidate for developing the insect virus production *in vitro* for controlling insect pests.

Key words: *Ac*MNPV, crude extract, cytotoxicity test, by 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay.

INTRODUCTION

In Thailand, *Stemona* species are known as Non Tai Yak. *Stemona* spp. can be used as insect repellent and insecticides such as scabicide and pediculocide (Greger, 2006; Chanmahasathien et al., 2011). The total alkaloid

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profiles could be grouped into four types represented as the major component by stenine-type *Stemona* alkaloids such as tuberostemonine and neotuberostemonine or by non-stenine types such as croomine and stemoninine. The biological activities of *Stemona* alkaloids were insecticidal and larvicidal activities (Brem et al., 2002; Kaltenegger et al., 2003; Xu et al., 2006).

For the replication of baculovirus in insect cell culture, there are three phases in the infection cycle: early (reprogramming the cell for virus replication), late (producing BV) and very late (producing ODV) (O'Rielly et al., 1992). The transition from the early phase to the late phase of infection is dependent upon viral DNA replication and occurs between 6 and 12 h after the initiation. During the late phase of infection, newly replicated viral DNA is condensed and packaged within the nucleus, in association with the virogenic stroma, into capsid structures to form nucleocapsids. From about 12 to 20 h, these nucleocapsids leave the nucleus, travel through the cytoplasm, and bud through a modified plasma membrane to acquire a loosely fitting envelope important for BV infectivity. Beginning at about 20 h, there is a transition from the late phase to very late phase of infection, nucleocapsids remain within the nucleus, become bundled together, and are enveloped by a membrane elaborated within the nucleus (Olszewski and Miller, 1997). The BVs enter insect cells by endocytosis that include: (1) virion binding to a host cell receptor, (2) invagination of the host plasma membrane, (3) formation of endocytic vesicle containing the enveloped virion, (4) acidification of the endosome, (5) activation of the viral envelope fusion protein, (6) fusion of the viral and endosomal membranes, and (7) release of the viral nucleocapsid into the cytoplasm (Blissard, 1996). The *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV), which is the type species and the most widely studied of the Baculoviridae (Wu et al., 2006). It has a wide host range, replicates well in the commonly used insect cell culture systems (*Spodoptera frugiperda*) and there is a wide range of commercially available transfer vectors (Hitchman et al., 2009). The size of *Ac*MNPV is 25×250 nm, and contains approximately 128 kbp double-stranded DNA (Arif, 1986). The replication of *Ac*MNPV occurs in the nuclei of infected cells and takes place in two phases. In the first phase, nucleocapsids are formed in the nucleus. These nucleocapsids reach the cytoplasm by passing through nuclei pores. The nucleocapsids gain envelope during the budding through the plasma membrane, and the particles released from the cell (Fraser, 1986). In the second phase, after nuclocapsids acquire envelope (apparently *de novo*) within the nucleus, viral occlusion bodies of NPV are known as polyhedral inclusion bodies (PIBs), and there are infective particles among insect in nature (Bilimoria et al., 1992).

The extract of *Stemona tuberosa* roots was found to have antibacterial, antifungal and insecticidal activities. Thus, the objective of this study was to evaluate the cytotoxicity of crude hexane, dichloromethane and ethanol extracts of *S. tuberos*a roots against insect cell line (Sf9) using MTT assay. The crude extract that showed the highest cytoxicity to Sf9 cells was to investigate the efficiency of the extract on the replication of insect virus (*Ac*MNPV) using the endpoint dilution assay.

MATERIALS AND METHODS

Preparation of *S. tuberosa* extracts

Fresh *S. tuberosa* roots were purchased from Chatuchak market in Bangkok, Thailand. Fresh roots were dried at 45°C until they were quite dried and were ground to powder using a milling machine. The powdered plant material (30 g) was macerated for 7 days sequentially with 450 ml of the following solvents: hexane, dichloromethane and ethanol. The hexane, dichloromethane and ethanol extracts were carefully filtered through Whatman® No. 1 filter paper and were evaporated to dryness under reduced pressure using rotary evaporator to give 0.155, 0.256 and 0.596 g, respectively.

Cell line and virus

The *S. frugiperda* cell line (Sf9) was used as the host cell for virus infection. Cells were cultivated in TNM-FH medium supplemented with 5% fetal bovine serum (FBS), 100 units/ml penicillin G, 100 µg/ml streptomycin in 25 cm^2 culture flasks at 28°C (Petcharawan et al., 2009; Petcharawan et al., 2012). The *Ac*MNPV stock was prepared by inoculating in Sf9 cells and incubated at 28°C. The virus suspension was removed seven days post infection and the virus titer of stock was determined as $TCID_{50}$/ml (tissue culture infectious dose per ml) and converted to pfu/ml (plaque forming unit per ml) (Reed and Muench, 1992), virus stock was stored at -20°C until needed.

Cytotoxicity assay

The extracts of *S. tuberosa* root were tested for *in vitro* cytotoxicity, using Sf9 cell line by 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Each crude extract was separately dissolved in 1 ml of dimethyl sulfoxide (DMSO) and volume was made up to 10 ml with maintained medium to obtained a stock solution of 5 mg/ml concentration, sterilized by filtration to make stock solution, then diluted to various concentrations (2,000, 1,000, 500, 250, 125, 62.5, and 31.25 µg/ml) in TNM-FH medium. The cells were seeded in a 96-well plate with 2×10^4 cells/well and incubated at 28°C for 24 h. After 24 h of seeding, the culture medium was replaced with fresh medium containing the different concentrations of the extracts. Each concentration was applied in 4 wells and within 3 independent experiments. Control cells were incubated without the extracts. After incubation at 28°C for 48 and 96 h, the MTT (10 µl of 5 mg/ml in PBS) was added in each well and the cells were incubated at 28°C for 4 h. Thereafter, the medium with MTT was removed from the wells and formazan crystal formed was dissolved by adding 150 µl/well a mixture of 10% SDS:DMSO (1:9). The plates were then shaked for 5 min and the absorbance of the solution in each well was measured at 570 nm on a microtiter plate reader (Siddiqui et al., 2010).

The percentage of cytotoxicity was calculated as {(A-B)/A}×100, where A and B are the absorbance of the control cells and treated

cells, respectively. All absorbance values were corrected against blank wells which contained only growth medium. For the evaluation of the concentration that induced 50% cytotoxicity relative to controls (CC_{50} values) was calculated in each assay using GraphPad Prism software program (Chiba et al., 1998; Krzyminska et al., 2010; Duman, 2012; Petcharawan et al., 2012).

Effect of crude extracts on the replication of *Ac*MNPV

The effect of *S. tuberosa* crude extract on the replication of *Ac*MNPV was conducted using cell monolayers in 35-mm tissue culture dishes. Exponential phase Sf9 cells in TNM-FH medium were seeded into a 35-mm tissue culture dish at a density of 4×10^5 cells/dish and incubated at 28°C overnight. The medium in each dish was aspirated and carefully replaced with 1 ml of *Ac*MNPV at a multiplicity of infection (MOI) of 2. After inoculation of virus, the dishes were placed on a rocker platform and rocked for 1 h. The inoculum was removed and washed with PBS, then fresh medium was added to each dish. At this time, it was considered as 0 h post infection (p.i.). The dishes were incubated at 28°C for 4 days. At the end of the incubation period, the cells were scraped and counted, and then centrifuged at 3,500 rpm for 15 min to separate the supernatant and the pellet. The supernatant that contained budded virion was kept at -20°C until needed. The pellets were resuspended with 0.1% sodium dodecyl sulfate (SDS) and incubated at room temperature overnight, then suspended cells were centrifuged and the SDS was discarded. The pellets were resuspended in 2 ml of sterile distilled water and counted the number of polyhedra that liberated from the infected cells in the control and the test samples. Each panel included toxicity control (cells incubated with the extract), uninfected cell control (cells incubated with medium) and infected cell control (cells incubated with virus) and the corrected % mortality was calculated using Abbot's formula, corrected % mortality = 100×{(T% - C%)/(100% - C%)}, where T% = the percentage of dead test cells and C% = the percentage of dead control cells (Abbot, 1925; Undeen and Vávra, 1997).

Finally, the virus concentration was determined as $TCID_{50}$/ml and converted to pfu/ml (Reed and Muench, 1992). The percentage of infected cells, the number of occlusion bodies (OBs)/ml in the control and the test samples were determined. The percent of reduction in the pfu/ml produced by virus in the presence of extracts was calculated as % reduction = 100 - [(pfu at given extract dose/pfu in control) × 100] (Sökmen, 2001).

Statistical analysis

The program GraphPad Prism 5.0 was used for the calculation of cytotoxicity curves and CC_{50} (Motulsky, 2007). All the data were statistically evaluated with SPSS statistics 17.0 software. Hypothesis testing methods include one way analysis of variance (ANOVA) followed by Duncan's new multiple range test, a post hoc or multiple comparison test. A value of $p<0.05$ was regarded as statistically significant differences (Custódio et al., 2011).

RESULTS

The effect of crude hexane, dichloromethane and ethanol extracts from *S. tuberosa* against Sf9 cell line was evaluated using MTT assay. Results of the cytotoxicity evaluation of these extracts against Sf9 cell line are shown in Table 1. The dichloromethane extract was more toxic than the others. The values of CC_{50} of these extracts against Sf9 cell line after 48 and 96 h of exposures are shown in Table 2. Based on 96 h exposure results obtained from hexane, dichloromethane and ethanol extracts from *S. tuberosa* against Sf9 cell line, the CC_{50} values were >2,000, 1,708.98, and >2,000 µg/ml, respectively. Among the crude extracts tested, the dichloromethane extract gave the highest toxic to Sf9 cells. However, the cytotoxicity of these extracts can be categorized using the classification of the cytotoxicity of natural ingredients reported by Gad Shayne (1999) and Shirazi et al. (2004), we can conclude that these extracts had partially nontoxic to Sf9 cell line, because of the CC_{50} were more than 1,000 µg/ml (Table 2).

The results (Table 3 and Figure 1) showed that there was no significant difference ($p>0.05$) between the percentage of infected cells in the control sample (95.54%±3.18) and in the test sample with crude dichloromethane extract (98.51%±1.34). However, there was significant difference ($p<0.05$) between the average virus titer in the control (2.06×10^8±0.71 PFU/ml) and the test (2.65×10^8±0.79 PFU/ml) samples. The average number of polyhedra (OBs/ml) in the control (5.11×10^6±0.63 OBs/ml) was not significantly different ($p>0.05$) from the average number of polyhedra in the test sample (4.19×10^6±0.31 OBs/ml). However, there was significant difference ($p<0.05$) between the percent reduction of virus titer in the control (0%±0) and in the test samples (-27.45%±4.36). These results showed that the crude dichloromethane extract of *S. tuberosa* at the concentration of 31.25 µg/ml was no toxic effect to Sf9 cells and could not inhibit *Ac*MNPV infection cultivated *in vitro* but it could enhance the virus titer (budded virus, BV) of *Ac*MNPV in Sf9 cells.

DISCUSSION

According to the results of this study, the crude dichloromethane extract of *S. tuberosa* root was more toxic to Sf9 cells than crude hexane and ethanol extracts, with the CC_{50} values of 1708.98, 2,000 and 2,000 µg/ml, respectively. The result corresponded with the report of Phattharaphan et al. (2010), the highest insecticidal activity to the third instar larvae of *Plutella xylostella* was observed from dichloromethane extract of *Stemona collinsae* root with the LC_{50} of 0.71% and the major active compound responsible for the insecticidal activity was hydroxystemofoline (alkaloid). Brem et al. (2002) reported that tuberostemonine was dominating alkaloid in the roots of *S. tuberosa* Lour., showing repellency but not toxic effect to neonate larvae of *Spodoptera litoralis*. Lee et al. (2006) reported that three new bibenzyl glycosides were isolated from *S. tuberose* significantly protected human neuroblastoma SH-SY5Y cells from 6-hydroxy dopamine induce neurotoxicity.

In order to determine the antiviral effect of crude dichloromethane extract of *S. tuberosa* root, the experiment was performed with the concentration of

Table 1. Comparison of the cytotoxic effect of crude hexane, dichloromethane and ethanol extracts on Sf9 cell line after 48 and 96 h of exposures.

Solvents	Concentration (µg/ml)	Mean percent cytotoxicity±SD*	
		48 h	96 h
Hexane	2000	19.86 ± 1.35^{cd}	41.05 ± 1.17^{b}
	1000	18.03 ± 1.29^{de}	31.08 ± 0.93^{c}
	500	11.65 ± 0.91^{fg}	19.88 ± 2.17^{efg}
	250	10.12 ± 0.51^{gh}	17.19 ± 2.12^{fgh}
	125	9.49 ± 0.30^{ghi}	14.08 ± 3.20^{gh}
	62.5	5.67 ± 0.61^{jk}	12.50 ± 1.88^{hi}
	31.25	3.13 ± 1.38^{kl}	1.15 ± 0.13^{jk}
	1% DMSO	1.54 ± 0.49^{lm}	1.32 ± 0.39^{ijk}
	0 (Control)	0 ± 0^{m}	0 ± 0^{k}
Dichloromethane	2000	42.68 ± 1.61^{a}	52.80 ± 2.13^{a}
	1000	35.03 ± 4.55^{b}	39.43 ± 4.24^{b}
	500	21.29 ± 0.60^{c}	29.82± 5.28^{cd}
	250	16.12 ± 0.36^{e}	25.20 ± 6.85^{cde}
	125	11.69 ± 0.44^{fg}	20.74 ± 8.60^{ef}
	62.5	7.68 ± 0.18^{hij}	17.23 ± 10.11^{fgh}
	31.25	2.25 ± 0.73^{lm}	7.67 ± 0.77^{i}
	0.4% DMSO	1.35 ± 0.02^{lm}	1.22 ± 0.10^{jk}
	0 (Control)	0 ± 0^{m}	0 ± 0^{k}
Ethanol	2000	20.15 ± 2.14^{cd}	41.04 ± 2.59^{b}
	1000	16.23 ± 4.30^{e}	30.84 ± 3.24^{c}
	500	13.43 ± 2.68^{f}	24.17 ± 1.33^{de}
	250	8.97 ± 0.30^{ghi}	21.96 ± 1.14^{ef}
	125	6.81 ± 0.83^{ij}	16.41 ± 1.21^{fgh}
	62.5	5.82 ± 0.89^{j}	7.25 ± 0.31^{ij}
	31.25	1.99 ± 1.40^{lm}	0.47 ± 0.44^{k}
	0.2% DMSO	0.81 ± 0.56^{lm}	0.48 ± 0.45^{k}
	0 (Control)	0 ± 0^{m}	0 ± 0^{k}

*Means within the column followed the same letter is not significant difference (p<0.05, Duncan's new multiple range test).

Table 2. The values of CC_{50} of crude extracts from *S. tuberosa* root against Sf9 cell line after 48 and 96 h of exposures.

Solvents	The incubation time (h)	Mean CC_{50} (µg/ml)
Hexane	48	>2000
	96	>2000
Dichloromethane	48	>2000
	96	1,708.98
Ethanol	48	>2000
	96	>2000

31.25 µg/ml because of this concentration did not have any effect on the growth of Sf9 cells. When the extract was added 1 h after infection with *Ac*MNPV, the virus titer was increased and the polyhedra or occlusion bodies

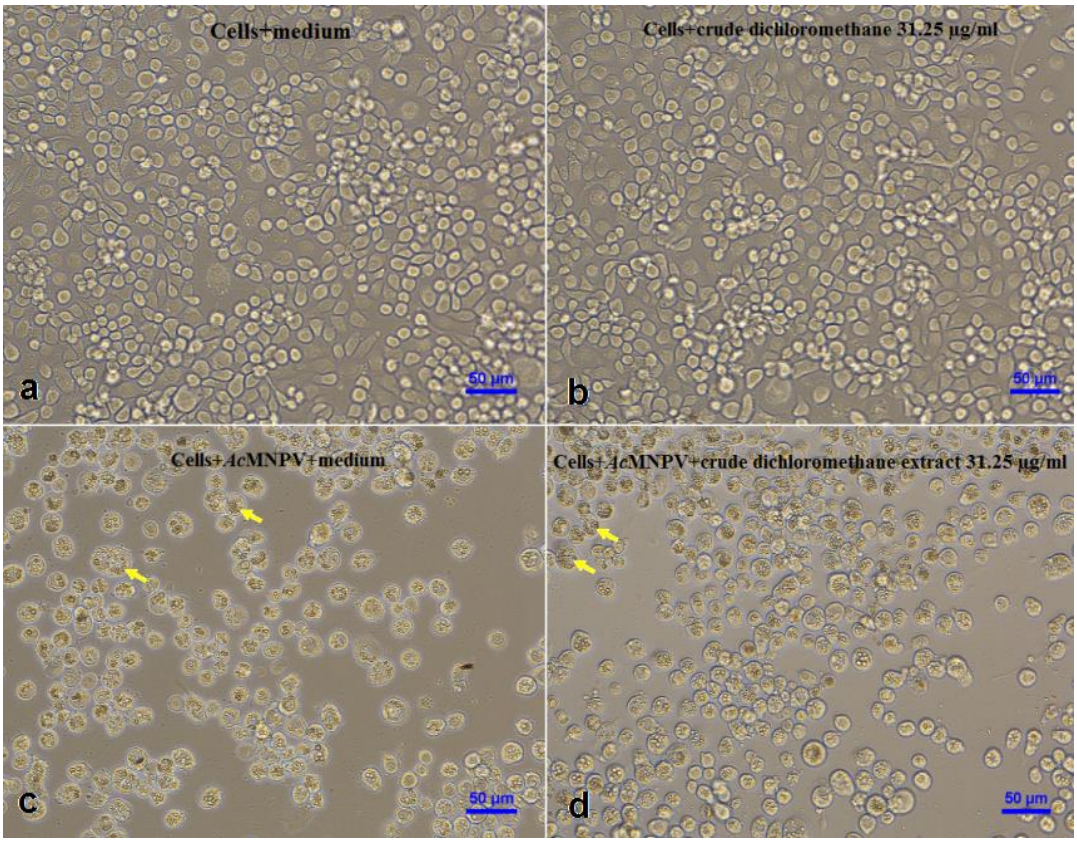

Figure 1. Phase-contrast micrographs of Sf9cells cultivated in TNM-FH medium (a), Sf9 cells after 96 exposure in dichloromethane extract (b), Sf9 cells after 96 h post infection with *Ac*MNPV in culture medium (c), Sf9 cells after 96 h post infection with *Ac*MNPV in 31.25 µg/ml of extract (d).

Table 3. Effect of crude dichloromethane extracts from *S. tuberosa* roots (31.25 µg/ml) on the replication of *Ac*MNPV.

Treatments	*Mean % infectivity	*Mean no. of OBs/ml (×10^6)	*Mean virus titer (PFU/ml) ± SD (×10^8)	*Corrected % reduction of budded virus
Cells+AcMNPV	95.54±3.18^a	5.11±0.63^a	2.06±0.71^a	0±0^a
Cells+AcMNPV+extract	98.51±1.34^a	4.19±0.31^a	2.65±0.79^b	-27.45±4.36^b

*Different letters indicate statistically significant differences between rows in the same column, p<0.05.

(OBs) could be produced in the nucleus of Sf9 cells (Table 3). The results indicated that this extract could not inhibit the viral synthesis, the viral structural and the viral occlusion protein phases. Further studies are necessary to investigate the mechanism of crude dichloromethane extract of *S. tuberosa*, such as the enhancement activity of *Ac*MNPV, purification and active compounds. These results corresponded with the report of Erturk et al. (2000), the effect of *Nerium oleander* L., *Prunus laurocerasus* L., *Punica granatum* L., *Olea europaea* L. and *Daphne glomerata* Lam. extracts could increase the concentration of progeny virus of AcMNPV.

This study showed that *S. tuberosa* can be a good candidate for developing the insect virus production *in vitro* for controlling insect pests.

Conflicts of Interests

The authors have not declared any conflict of interests.

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Cytotoxic, antibacterial and antibiofilm activities of aqueous extracts of leaves and flavonoids occurring in Kalanchoe pinnata (Lam.) Pers.

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Several investigations have demonstrated *in vitro* antibacterial activity of plant extracts. *Kalanchoe pinnata* is globally used in folk medicine for the treatment of various diseases, such as diarrhea, infections, tuberculosis and fever. Among the enteropathogenic bacteria, enteroaggregative *Escherichia coli* (EAEC) is an important cause of diarrhea in the world. EAEC is also capable of forming biofilms, conformation that provides antibiotic resistance and a high degree of dispersion and reinfection, which may represent up to 80% of causes of human microbial infections. Thus, it becomes necessary to search for new antimicrobial agents with activity against biofilms. The aim of this study was to evaluate the cytotoxic, antibacterial and antibiofilm in EAEC from aqueous extracts of leaves and the flavonoids quercetin and rutin, occurring in *K. pinnata*. Leaf aqueous extracts were obtained and evaluated phytochemically. The cytotoxic activity was evaluated in human carcinoma cell lines HEp-2, Caco-2 and T84. The antibacterial activity was evaluated by the macrodilution method and the evaluation of antibiofilm activity was performed in *Escherichia coli* enteroaggregative (EAEC 042). Aqueous extracts of *K pinnata* do not show toxicity to Hep-2, but all other cell lines were sensitive to this extract. Instead, the flavonoids quercetin and rutin showed no cytotoxicity with any of the tested cell lines. Quercetin is capable of inhibiting bacterial growth of all tested strains. The aqueous leaf extract and quercetin were able to inhibit EAEC 042 biofilm formation above 50%. The results indicate the potential use of the species in treatment of bacterial infections.

Key words: Antimicrobials, EAEC 042, biofilms, quercetin, Crassulaceae.

INTRODUCTION

Kalanchoe pinnata (Lam.) Pers., also referred by its synonym *Bryophyllum pinnatum* (Lam.) Kurz., is a species of the Crassulaceae family, commonly known in Brazil as saião-roxo (Oliveira et al., 2003). This plant

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is commonly used for various medical applications, such as in the treatment of diarrhea, conjunctivitis, dermatitis, eczema, fever, infections, tuberculosis, respiratory infections, inflammation, among others (Majaz et al., 2011).

The *in vitro* antibacterial activity of various extracts from *K. pinnata* had been reported in the literature, like methanolic and alcoholic extracts from leaves, stem and roots. Among the bacterial species susceptible to these extracts are *Enterococcus faecalis* (Aibinu et al., 2007), *Escherichia coli* (Pattewar et al., 2013), *Klebsiella pneumoniae* (Okwu and Nnamdi, 2011), *Pseudomonas aeruginosa* (Tatsimo et al., 2012), *Salmonella typhi* (Nwadinigwe, 2011), *Shigella dysenteriae* (Akinpelu, 2000), *Staphylococcus aureus* (Biswas et al., 2011), and others. Among the enteropathogenic bacteria, enteroaggregative *Escherichia coli* (EAEC) is an important cause of diarrhea in the world, being related to cases of diarrhea of persistent diarrhea especially in children, HIV-infected individuals and from travelers in developing countries (Berry et al., 2014). In these countries, the cause of death due to persistent diarrhea in infants between 1 and 11 months is 30%. In children up to 4 years old this rate can reach 69% (Rahman et al., 2014).

In addition to its aggregative ability in human intestinal cells, EAEC is able to form biofilms on surfaces. This configuration provides antibiotic resistance and a high degree of dispersion and reinfection, which may represent up to 80% of causes of human microbial infections (Bueno, 2014). The nature of biofilms is able to protect them from adverse conditions such as desiccation, UV, toxic compounds and antibiotics, favoring the persistence (Abdel-Aziz and Aeron, 2014). Thus, is it important to search for new antimicrobial agents with activity against biofilms.

Much of the antimicrobial activity of plant extracts is related to the action of flavonoids, substances that also have nutraceutical importance, due to the organoleptic properties of food and pharmacological activities on human health (Tapas et al., 2008).

Although not produced by the human metabolism, flavonoids have various pharmacological activities such as anti-allergic, anti-inflammatory and anti-ulcer. Over four thousand substances belonging to the group have been identified, having highlighted in *K. pinnata*, the compounds of the group of flavonols, such as quercetin and rutin (Lopes et al., 2000).

For quercetin, important antimicrobial properties have been identified, such as antileishmanial (Muzitano et al., 2006, Muzitano et al., 2011) and antibacterial (Gatto et al., 2002, Mittal et al., 2014; Prasad et al., 2014), being a metabolite of great interest in antimicrobial studies.

Thus, the objective of this study was to evaluate the cytotoxic activity in different cell lines, antibacterial activity in different strains and antibiofilm in EAEC from aqueous extracts of leaves and the flavonoids quercetin and rutin, occurring in *K. pinnata*.

MATERIALS AND METHODS

Plant material

Samples of *K. pinnata* were collected in Jacarepaguá (Rio de Janeiro/Brazil) and a voucher specimens was deposited in the Herbarium of the University of the State of Rio de Janeiro (UERJ) under the number HRJ12515.

For the different analyses, samples of *K. pinnata* were grown in the greenhouse at UERJ. Plants were cultivated under *in vivo* conditions, in containers using fertilized soil, under sunlight and were watered twice a day. Samples of material were collected in the summer, between the months of November 2014 and February 2015, for the production of extracts and phytochemical analysis.

Phytochemical analysis

For the phytochemical analysis and evaluation of the medicinal potential of *K. pinnata*, aqueous extracts were prepared from leaves according to the methodology proposed by Muzitano et al. (2011).

This fresh material was mechanically macerated and heated in distilled water at 20% (w/v) for 30 minutes at 50°C. After this period, the material was filtered, frozen at -20 and then lyophilized under pressure of 60 µmHg at -59°C. After lyophilization, the material was resuspended in sterile distilled water at the concentrations required for experimentation.

The flavonoids quercetin and rutin (Sigma-Aldrich) were used as standard substances for analysis, being solubilized 1 $mg.mL^{-1}$ of substance in methanol (MeOH).

The qualitative analysis for the phytochemical aqueous extracts was performed following the protocol proposed by Barbosa (2001). Groups of secondary metabolites of medicinal interest were evaluated, including flavonoids, alkaloids, saponins, phenols and tannins.

Cytotoxic activity of *K. pinnata* extracts

In order to evaluate the cytotoxic activity, cell lines that represent cells related to passage of aqueous extract in the gastrointestinal tract were selected. Thus, the cell lines HEp-2 (ATCC CCL23 originating from human larynx carcinoma), Caco-2 (ATCC HTB37, originating from human colon adenocarcinoma) and T84 (ATCC CCL248 originating colon rectal carcinoma) were used in the study.

The aqueous extracts were evaluated at concentrations of 500 and 1000 $\mu g.mL^{-1}$ and the flavonoids quercetin and rutin at the concentration of 50 $\mu g.mL^{-1}$. The assay was performed in 96-well plates showing confluent layer of cells and cultivated in 100 µL of DMEM or MEM medium per well. In each culture well 100 µL extract or flavonoids solubilized in PBS-D were added, keeping the culture for 24 h at 37°C and 5% of CO_2.

In the corresponding period of three hours before the end of cultivation (45h) 5 $mg.mL^{-1}$ of solution of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was added. After this period, the plate was centrifuged at 3000 rpm for 5 min to deposition of MTT crystals formed. The supernatant was discarded and the crystals diluted by adding 100 µL of DMSO (100%) in each well.

The reading was performed in a microplate reader at 492 nm. As

negative controls, cell cultures were used in the absence of treatment and cultivation in the presence of 5% methanol. As a positive control, cells were cultured in the presence of 100% methanol. The experiments were performed in triplicate and repeated three times or more.

Antibacterial activity

In all assays, reference strains were used, obtained mostly from the American Type Culture Collection (ATCC, Rockville, MD, USA) and the other, of the collection of the Department of Microbiology, Immunology and Parasitology, UERJ (Table 1).

The evaluation of the antimicrobial activity of the aqueous extract and flavonoids has been carried out by the method of agar dilution (macrodilution) used by Soberon et al. (2007) with some modifications.

The bacterial strains were grown in 15×100 mm test tubes containing 3 ml of Mueller- Hinton Broth (MHB, Oxoid, Ltd.), incubated at 37°C for 18 h. The extract, at 1000 µg.mL^{-1} and the flavonoids, at the concentrations of 10 and 100 µg.mL^{-1} were solubilized in 20 ml of Mueller-Hinton Agar (MHA), not yet solidified, and poured into 90 mm petri dishes. As negative controls of the experiment were used MHA medium and the same medium plus 1 and 10% of MeOH representing the final concentration of solvent after solubilization of quercetin and rutin.

For each treatment, 2 µL of each bacterial suspension in duplicate were plotted on the plate. The growth of colonies was evaluated after 24 h incubation at 37°C. All the material and culture media were sterilized by autoclaving at 121°C for 20 min. The experiments were repeated three times or more.

Evaluation of biofilm formation of enteroaggregative *Escherichia coli* (EAEC 042)

The evaluation of the growth of biofilms of EAEC 042 was performed based on the spectrophotometric assay of biofilm inhibition, using as reference the working of Namasivayam and Roy (2013) with some modifications.

E. coli suspensions were adjusted and standardized to 0.14 nm of optical density (OD), and added 100 µL of the suspension in 96 well plates. Then the extract and flavonoids was added in triplicate, being the culture maintained in incubation for 18 h at 37°C. The aqueous extract was evaluated at 1000 µg.mL^{-1} and the flavonoids quercetin and rutin in concentrations of 5 and 50 µg.mL^{-1}.

After incubation, the supernatant was discarded and added 100 µL of crystal violet solution (1% w/v) for 30 min. After this period, the solution was removed and wells were washed with PBS-D, and then added 100 µL of 95% ethanol, keeping incubation for 15 min for solubilization of the crystals. The reading was performed in microplate reader at 570 nm. The calculation of the percentage inhibition of biofilm formation was in accordance with the formula:

$$\% \text{ of inhibition} = \frac{\text{OD in control - OD in treatment} \times 100}{\text{OD in control}}$$

Statistical analysis

The experiment with comparisons between controls was performed by the analysis of variance of the means obtained (ANOVA) using the program GraphPad PRISM®, being carried out after Bonferroni post-test, with $p<0.05$.

RESULTS

Phytochemical analysis

Phytochemical analysis of *K pinnata* leaf extracts used in the experiments (Table 2) showed the presence of flavonoids, saponins, phenols and tannins.

Cytotoxic activity of *K. pinnata* extracts

In cell cultures of HEp-2 (Figure 1a) the aqueous extract of leaves showed no toxicity. However, Caco-2 and T84 strains (Figure 1b and c) were more sensitive to the culture in the presence of the extract in a high concentration (1000 µg.mL^{-1}).

The flavonoids quercetin and rutin and the methanol at a concentration of 5% showed no toxicity for the cultivation of different cell lines evaluated.

Antibacterial activity

The aqueous extracts of *K. pinnata* showed antibacterial activity, reducing the growth of *Citrobacter freundii*, *Bacillus thuringiensis*, *Shigella sonnei* and *Escherichia coli* K-12 (HB 101) grown in the presence of aqueous leaf extract obtained from plants grown *in vivo* (Table 2).

At 10 µg.mL^{-1}, rutin was able to reduce the growth of *Pseudomonas aeruginosa* and *C. freundii*. Have quercetin in same concentration reduced the growth of *P. aeruginosa*, *C. freundii*, *E. coli* (17-2), *Serratia marcescens* and *B. thuringiensis*. When the concentration of these flavonoids is raised to 100 µg.mL^{-1} in culture, the rutin is able to reduce the growth of all strains with the exception of *Enterococcus faecalis*, whereas quercetin inhibited the growth of all analyzed strains (Table 3).

The controls using methanol are related to the final concentration of solvent in the plate containing quercetin or rutin, due to the fact of these substances are soluble only in this condition. At 10%, methanol in culture showed influence on the growth of *P. aeruginosa*, *C. freundii*, *E. coli* (17-2), *E. coli* (ATCC 25922) and *Staphylococcus aureus*.

Therefore, results of reduction or inhibition of growth of these strains in the presence of 100 µg.mL^{-1} of rutin and quercetin cannot be considered due to the possible influence of the solvent in cultivation. Conversely, such as 10% methanol in culture has no influence on the growth of other strains, these results can be validated.

Antibiofilm activity

The formation of biofilms of enteroaggregative *E. coli*

Table 1. Strains used in the evaluation of the antibacterial potential of *Kalanchoe pinnata*.

Gram-positive		Gram-negative	
Species	**Strain**	**Species**	**Strain**
Bacillus thuringiensis	ATCC 33679	*Citrobacter freundii*	ATCC 12241
Enterococcus fecalis	29212	*Escherichia coli*	EAEC 17-2
Staphylococcus aureus	ATCC 25923	*Escherichia coli*	ATCC 25922
Staphylococcus saprophyticus	ATCC 15305	*Escherichia coli*	ATCC 35218
Staphylococcus simulans	ATCC 27851	*Escherichia coli*	042
Streptococcus pyogenes	8668	*Escherichia coli* K-12	C600
		Escherichia coli K-12	HB 101
		Klebsiella pneumoniae	ATCC 700603
		Pseudomonas aeruginosa	ATCC 27853
		Salmonella enterica Typhimurium	C20
		Serratia marcescens	7145
		Shigella sonnei	ATCC 25931

Table 2. Phytochemical screening of the aqueous extract of *K. pinnata* leaves.

Secondary metabolites	Presence in the extract
Flavonoids[1]	+
Alkaloids[2]	-
Saponins	+
Phenols[3]	+
Tannins[3]	+

+ = presence of the compound, - = absence of the compound, [1]Test for flavones, flavonols and xanthones, [2]Tests with reactive of Meyer and Bouchardat, [3]Test with alcoholic solution of ferric perchlorate.

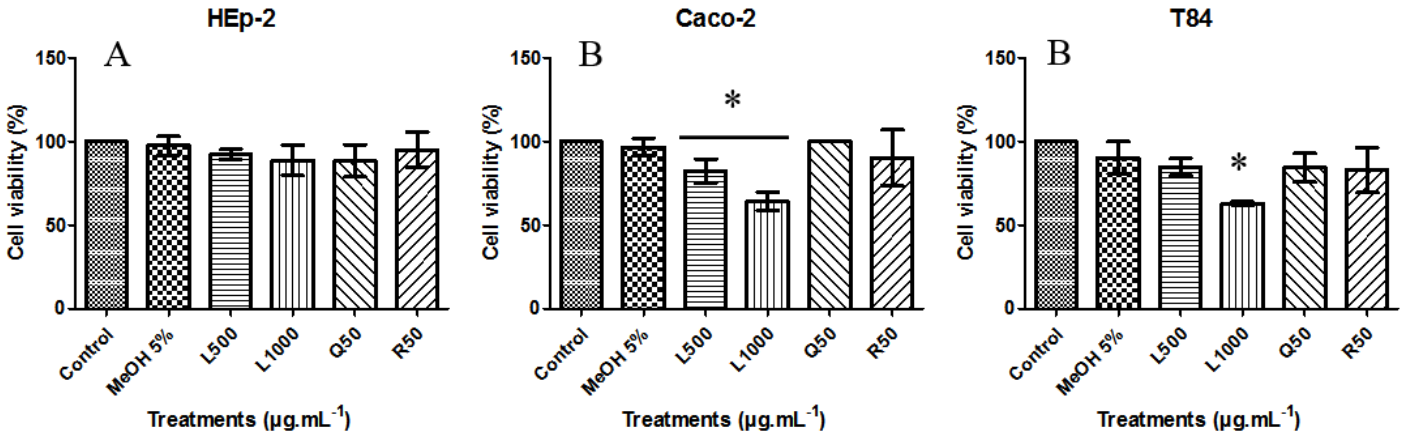

Figure 1. Cytotoxicity of aqueous extracts of leaves and flavonoids occurring in *Kalanchoe pinnata* after 24 h of culture at 37 and 5% CO2. A - HEp-2; B - Caco-2; C - T84. L = leaf extract, Q = quercetin, R = rutin, * = statistically different at the Bonferroni test ($p<0.05$).

(EAEC 042) was altered when grown in the presence of aqueous extract of leaves from *K. pinnata* and quercetin (Figure 2). Although not express inhibiting the growth of this strain in the macrodilution assay (Table 3), the aqueous extracts of the leaves were able to reduce the formation of biofilms of the EAEC 042 exceeding 50%.

Table 3. Bacterial growth in the presence of flavonoids quercetin and rutin and aqueous extracts of leaves (µg.mL^{-1}) of *K. pinnata*.

Strain	**L** 1000	**RU** 10	**Q** 10	**MeOH 1%**	**RU** 100	**Q** 100	**MeOH 10%**
P. aeruginosa (ATCC 27853)	-	R	R	-	R*	I*	R*
C. freundii (ATCC 12241)	R	R	R	-	R*	I*	R*
*Enteroaggregative E. coli (*EAEC 17-2*)*	-	-	R	-	R*	I*	R*
E. coli (ATCC 25922)	-	-	-	-	R*	I*	R*
S. aureus (ATCC 25923)	-	-	-	-	R*	I*	R*
S. marcescens (7145)	-	-	R	-	R	I	-
S. simulans (ATCC 27851)	-	-	-	-	R	I	-
B. thuringiensis (ATCC 33679)	R	-	R	-	R	I	-
S. saprophyticus (ATCC 15305)	-	-	-	-	R	I	-
S. enterica Typhimurium (C20)	-	-	-	-	R	I	-
E. faecalis (29212)	-	-	-	-	-	I	-
E. coli K-12 (C600)	-	-	-	-	R	I	-
S. sonnei (ATCC 25931)	R	-	-	-	R	I	-
E. coli (ATCC 35218)	-	-	-	-	R	I	-
S. pyogenes (8668)	-	-	-	-	I	I	-
E. coli K-12 (HB 101)	R	-	-	-	R	I	-
K. pneumoniae (ATCC 700603)	-	-	-	-	R	I	-
*Enteroaggregative E. coli (*EAEC 042*)*	-	-	-	-	R	I	-

L = Leaf extract; **RU =** rutin; **Q =** quercetin; **R** = reduction; **I** = inhibition; trace = similar growth to the control in MHA.* = Unvalidated for the possible influence of the solvent.

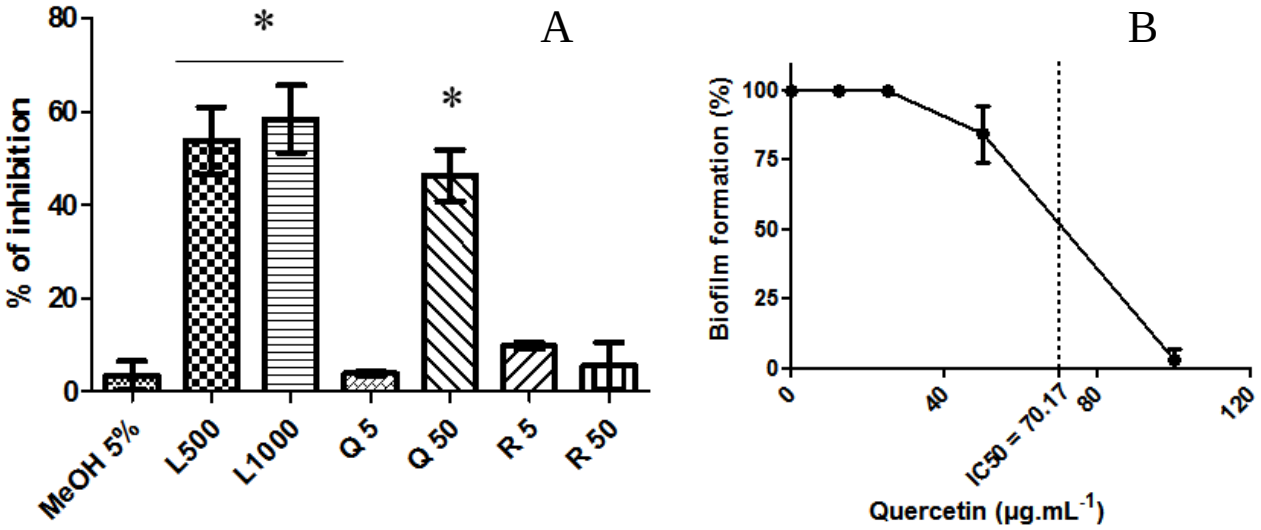

Figure 2. Biofilm formation of enteroaggregative *Escherichia coli* (EAEC 042). A, Activity of aqueous extracts of leaves and flavonoids occurring in *Kalanchoe pinnata* (µg.mL^{-1}) after 18 h of cultivation at 37°C. B, Inhibitory concentration of quercetin at 50% of biofilm formation. L = leaf extract, R = Rutin, Q = Quercetin. * = values are statistically different from the control to the Bonferroni test ($p<0.05$).

This reduction was observed in both tested concentrations (500 and 1000 µg.mL^{-1}).

At the highest concentration tested (50 µg.mL^{-1}), quercetin was able to reduce biofilm formation. For this compound, the inhibitory concentration for 50% of biofilm formation (IC50) was 70.17 µg.mL^{-1}. However, no inhibition was observed in the cultivation in the presence of rutin at concentrations of 5 or 50 µg.mL^{-1}.

DISCUSSION

The HEp-2 strain was resistant to the presence of the aqueous extract of *K. pinnata* in cultivation, which

was not observed for the other cell lines. The phytochemical analysis revealed the presence of flavonoids in this extract. Once cytotoxicity was not observed to any lineage to the flavonoids quercetin and rutin, the toxicity observed in the leaf extract may not be related to the free flavonoids present in the plant.

The cytotoxic activity of extracts of the species has been attributed to compounds called bufadienolides. These are characteristically present in leaves and stems of *K pinnata*, being demonstrated high cytotoxicity in human carcinoma lines, especially the compounds bryofilin B and bryofilin A (Milad et al., 2014). The presence of these compounds in the assessed aqueous extract may be related to the sensitivity observed in Caco-2 and T84 lines. This metabolite group can be considered the largest in this activity for this species, also having potential for chemoprevention of cancer (Supratman et al., 2001).

The aqueous extract of leaves showed a reduction in the growth of four of the evaluated strains. The antibacterial activity of aqueous extracts of *K. pinnata* leaves was also observed in *Propionibacterium acnes* (Kumar et al., 2013), *Alcaligenes faecalis* and *Bacillus subtilis* (Sharma et al., 2014).

As observed for the aqueous extract, *in vitro* studies have demonstrated the antibacterial activity of methanolic extracts of *K. pinnata* leaves. This action is observed in strains of genres evaluated in this study, as *E. faecalis* (Aibinu et al., 2007) *E. coli* (Pattewar et al., 2013), *K. pneumoniae* (Okwu and Nnamdi, 2011), *P. aeruginosa* (Tatsimo et al., 2012), *S. typhi* (Nwadinigwe, 2011), *S. dysenteriae* (Akinpelu, 2000), and *S. aureus* (Biswas et al., 2011).

The antibacterial activity in *S. marcescens* was also observed in methanol extracts of the plant (Sharma et al., 2014). In this work, the reduction and inhibition activities of this strain were observed only when grown in the presence of quercetin. The flavonoid may be one of the active principles present in methanolic plant extracts, since it shows high solubility in methanol (Megawati and Fajriah, 2013).

Quercetin has noted antibacterial activity at concentrations above 10 $\mu g.mL^{-1}$ being able to inhibit the growth of all the strains tested in this study. Besides these strains, quercetin also presents antibacterial activity in strains of *Proteus mirabilis*, *Acinetobacter baumannii*, *Helicobacter pylori* and also methicillin resistant *S. aureus* (MRSA) when evaluated alone or in combination with oxacillin (Ramos et al., 2006, Ozcelik et al., 2006).

Also present in *K. pinnata* extracts, rutin demonstrated antibacterial activity for all tested strains. The most significant result for this flavonoid was held at the concentration of 100 $\mu g.mL^{-1}$, with the reduction of all tested strains. In some strains, like *E. coli* and *S. aureus*, the minimum inhibitory concentration for rutin is presented in amounts exceeding 100 $\mu g.mL^{-1}$ (Araruna et al., 2012). This shows the lack of inhibitory activity in low concentrations of rutin, as well as 10 $\mu g.mL^{-1}$, evaluated in this study.

The antibacterial activity of aqueous extract of leaves of the species may also be related to the presence of other compounds derived from plant secondary metabolism, such as saponins, phenols and tannins, present in the extract evaluated in this study (Table 2).

The antibacterial activity of saponins isolated from plant extracts has been demonstrated in strains of *E. coli*, *B. subtilis* and others, as well as antifungal activity (Edewor et al., 2009, Kannabiran et al., 2009, Maatalah et al., 2012). Similarly, tannins and hydrolyzed tannins are active against the growth of *E. coli*, *B. subitilis*, *B. cereus* and *B. licheniformis*, *Shigella boydii*, *S. flexneri* and others, yeasts and fungi (Banso and Adeyemo, 2007, Lim et al., 2006). Antimicrobial activity of phenolic compounds, such as phenolic acids and coumarins, was observed in strains of *E. coli*, *Bacillus cereus* and fungi (Nohynek et al., 2006, Nitiema et al., 2012). The increase and the effectiveness of antibacterial activity of derivatives of phenolic acids are usually related to increasing the alkyl chain in the molecule, also the butyl ester regions being effective (Merkl et al., 2010).

Some phytochemical compounds have the ability to control the establishment and growth of bacterial biofilms. These compounds may act at different stages of biofilm formation, such as in bacterial adhesion, motility and "quorum-sensing", and have the advantage of having a lower probability of bacterial resistance (Borges et al., 2013). Besides acting in antimicrobial activity, plant extracts can be the basis for the synthesis of active compounds. *K. pinnata* leaf extracts can be used for the synthesis of silver nanoparticles with antibacterial and antibiofilm activity in *P. aeruginosa*, *S. aureus*, *S. pyogenes* and *Salmonella enterica* Typhi. This activity proves to be higher than that observed for ciprofloxacin (Phatak and Hendre, 2016).

As observed for EAEC, quercetin also shows antibiofilm activity to *S. aureus*, observed in *in vitro* assays. This quercetin in plant extracts is able to act in the repression of bacterial genes of cell adhesion and reduces the hemolytic capacity of *S. aureus*, being suggested for use in inhibiting the formation of recalcitrant biofilms (Lee et al., 2013).

It can be concluded that flavonoids, like quercetin, are active principles of the extracts of *Kalanchoe pinnata* for antibacterial activity. Thus, phytochemical studies aimed at the research and isolation of flavonoids of the species are promising in the search for new antimicrobial agents.

Conclusion

The aqueous extract of *K. pinnata* leaves at low concentrations and the flavonoids quercetin and rutin

showed no cytotoxicity in tested concentrations. The evaluated material presented antibacterial activity to various strains and potential to inhibit the formation of biofilms of EAEC 042. Quercetin must be considered as one of the active substance of interest responsible for the antibacterial activity.

Conflicts of Interests

The authors have not declared any conflict of interests.

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