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Full Length Research Paper

Comparative phytochemical studies and anti-inflammatory activities of *Desmodium velutinum* (Willd) and *Desmodium scorpiurus* Desv. (Family Papilionaceae) growing in Nigeria

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Members of the genus *Desmodium* are widespread in the sub-tropics, tropics and used in traditional medicine, as part of recipes for treating fever, pains, cough and dysentery. In this study, phytochemical screening and comparative anti-inflammatory activities of the methanol extracts, hexane and ethyl acetate fractions of the leaves of *Desmodium velutinum* and *Desmodium scorpiurus* were investigated using the egg-albumin induced oedema and protein denaturation methods. The presence of flavonoids, saponins, tannins and sterols were detected in the leaves of *D. velutinum* and *D. scorpiurus* while, the extracts and fractions exhibited anti-inflammatory activities not significantly different ($p < 0.05$) from the activities of the reference drug (Diclofenac Sodium). The hexane fractions of *D. velutinum* and *D. scorpiurus* exhibited the most pronounced effects of 76.9 and 68.2% respectively at a dose of 2000 µg/ml in the *in vitro* model; 27.2 and 24.5% respectively in the *in vivo* model. This study has been able to report the anti-inflammatory activities of the leaves of *D. velutinum* and *D. scorpiurus*.

Key words: *Desmodium velutinum, Desmodium scorpiurus,* anti-inflammatory activities, papilionaceae, *in vivo*, *in vitro*.

INTRODUCTION

The genus *Desmodium* is a member of the Papilionaceae family with more than three hundred and fifty (350) species widely spread and occurring throughout the sub-tropics and tropics (Lenne and Stanton, 1990). Traditionally, members of the genus *Desmodium* are used in the treatment of rheumatism, pyrexia, dysentery, cough, malaria and hepatitis while in the traditional Chinese medicine, most species of *Desmodium* are used in the treatment of fever, for neutralizing toxins, inhibiting pains and suppressing cough (Ma et al., 2011).

Previous biological studies have reported the antibacterial, hepatoprotective, anti-inflammatory, diuretic,

antipyretic analgesic and choleretic activities of *Desmodium* species (Liu et al., 2013) while, several flavonoids, alkaloids, terpenoids, steroids and a number of volatile oils have been characterized and isolated from about fifteen *Desmodium* species (Liu et al., 2013; Ma et al., 2011). However, most of the *Desmodium* species in Asia and Africa are yet to be fully studied in details.

D. *velutinum* is a perennial erect or sub-shrub which grows to about 3 m in height. It is used in folklore medicine for treating diarrhea, dysentery, stomachache (Setyowati-Indorto et al., 1999) and fever (Anowi et al., 2012) while, *D. scorpiurus*, commonly called Tick trefoil or Scorpion tick trefoil is a weak erect plant, used traditionally for the treatment of constipation, cough, convulsion, veneral infections and ringworm (Ndukwe et al., 2006). Though *Desmodium velutinum* and *Desmodium scorpiurus* are not documented for treating rheumatism and inflammation, the earlier reports of members of the genus *Desmodium* as part of recipes for treating inflammation (Liu et al., 2013) has led to investigating the anti-inflammatory activities and phytochemical analysis of these two species: *D. velutinum* and *D. scorpiurus* found growing in Nigeria.

MATERIALS AND METHODS

Plant collection and authentication

The leaves of *D. velutinum* and *D. scorpiurus* were collected from Eruwa near Ibadan in Oyo State in August, 2012 and authenticated at the Forestry Research Institute of Nigeria by Mr Chukwudi where, voucher specimens with voucher numbers FHI 108953 and 109646 were deposited.

Drying and extraction

The leaves of *D. velutinum* and *D. scorpiurus* were dried under shade and powdered in a miller. The powdered leaves were extracted by percolating in 80% methanol. They were filtered and the filtrates concentrated under reduced pressure to dryness after which a solvent-solvent partitioning was carried out successively with hexane and ethyl acetate to give the hexane and ethyl acetate fraction.

Phytochemical analysis

Phytochemical screening of the leaves of *D. velutinum* and *D. scorpiurus* were carried out using standard procedures (Evans, 2009).

Animals

Wistar albino male rats weighing between 130 and 150 g obtained from the Animal House of the University of Ibadan were allowed to acclimatize for two weeks before use and allowed 12 h Light and 12 h dark cycle at 25°C. Animals were handled according to the International standard guiding the handling of animals and fed with standard pellets and allowed water *ad libitum*.

Anti-inflammatory activities

In vivo assay

The egg-albumin model was used in the *in vivo* anti-inflammatory study as the phlogistic agent (Anosike et al., 2012). The initial diameters of the right hind paws were taken and rats grouped into five groups (n=5).

Group A – Rats were administered 200 mg/kg of extract or fraction
Group B – Rats received 100 mg/kg of extract or fraction.
Group C- Rats were administered 50 mg/kg of extract or fraction.
Group D – Rats were administered 5 mg/kg of Diclofenac (Cataphlam, Novartis)
Group E- Rats were administered 1 ml/kg of distilled water.

30 min after administering the extract, fraction or standard drug, 0.1 ml of egg albumin was injected into the right hind paw of each rat by sub plantar administration. Following the injection of the egg albumin, the hind paws of the rats were measured at an interval of 1 h for 6 h (0 to 6 h). The percentage inhibition of the edema was calculated as described by Jain and Khanna (1981).

$$\% \text{ Inhibition of oedema} = \frac{I_0 - I_1}{I_0} \times 100$$

I_0 = Change in paw circumference in control group; I_1 = change in paw circumference in treated groups.

In vitro anti-inflammatory assay

The *in vitro* anti-inflammatory assay was carried out using the method adopted from Sangita et al. (2012). 0.2 ml of egg albumin (from fresh hen's egg) was added to 2.8 ml phosphate buffer saline (PBS, pH 6.4) and 2 mls of the extract/fraction or standard drug using five doses (2000, 1000, 500, 250 and 125 µg/ml) while, equal volume of distilled water served as control. The varying mixtures were incubated at 37±2°C for 15 min after which, the mixtures were heated in a temperature regulated water bath at 70°C for 5 min. After cooling, the absorbance of the mixtures was read in the spectrophotometer at 660 nm and the vehicle used as blank. The percentage inhibition of protein denaturation was calculated as:

$\% \text{ PI} = 100 \text{ X } [1 - V_t]/V_c$
V_t = absorbance of test sample
V_c = absorbance of control.

Statistical analysis

The results are expressed as percentage inhibition (%) and data analyzed by using the one way analysis of variance (ANOVA) ($p<0.05$).

RESULTS AND DISCUSSION

The phytochemical screening of the leaves of *D. velutinum* and *D. scorpiurus* revealed the presence of flavonoids, saponins, tannins and sterols showing

Table 1. Phytochemical screening of *D. velutinum* and *D. scorpiurus* Leaves.

Qualitative analysis	*D. velutinum*	*D. scorpiurus*
Flavonoids	+	+
Saponins	+	+
Tannins	+	+
Alkaloids	-	-
Cardiac glycosides	-	-
Anthraquinones	-	-
Resins	+	-
Sterols	+	+

+ = Present; - = Absent.

similarities in the secondary metabolites they possessed (Table 1).

The phytochemical analysis of *D. velutinum* used in this study is however similar to the one collected from the South east region of Nigeria (Anowi et al., 2012). Furthermore, there were similarities in the secondary metabolites of the leaves of *D. scorpiurus* and *D. velutinum* as well as those *Desmodium* species previously studied (Ma et al., 2011; Liu et al., 2013).

The egg-albumin model used in this study is one of the models adopted for determining anti-inflammatory and anti- oedematogenic activities of medicinal plants (Hira et al., 2013). The model induces inflammation by producing histamine, serotonin and bradykinin in the first phase and the release of prostaglandins and nitric oxide in the second phase by showing peak inflammaton at 3 h (Seibert et al.,1994).

The methanol extracts, hexane and ethyl acetate fractions of *D. velutinum* and *D. scorpiurus* were investigated for their anti-inflammatory potentials. In the *in vivo* anti-inflammatory study, the activities of the methanol extract of *D. velutinum* leaf at 200 and 100 mg/ml were not significantly different ($p>0.05$) from each other but more pronounced than that of the reference drug (Diclofenac sodium) when compared with the untreated group. The methanol extract at 200 and 100 mg/ml exhibited a reduction in oedema of 28.8 and 32.1% at 2 h respectively while, the hexane fraction of the leaf of *D. veluti num* at 200 and 100 mg/kg exhibited same anti-inflammatory effect by reducing the oedema by 27.2% at 6 h (Tables 2 and 3). The ethyl acetate fraction exhibited a weak anti-inflammatory effect of 13.2 and 6.8% at 200 and 100 mg/ml after 6 h (Table 4).

The methanol extract of the leaf of *D. scorpuirus* exhibited dose dependent anti-inflammatory activities at all doses (200, 100 and 50 mg/ml) tested. The methanol extract at 200 and 100 mg/ml exhibited high anti-inflammatory effects of 27.1 and 21.9% 6 h after extract administration when compared to the untreated group (Table 5). The hexane fraction 6 h after administration at 200 and 100 mg/kg exhibited a reduction of 24.5 and 18.1% in the oedema and the activity of the ethylacetate was the weakest with a reduction of 18.1 and 14.3% at 6

h. This effect is similar with that exhibited by the ethylacetate fraction of the leaf of *D. velutinum.* In the *in vitro* assay, the methanol extracts, hexane and ethyl acetate fractions of *D. velutinum* and *D. scorpiurus* exhibited dose-dependent activities.

The hexane fractons of *D. velutinum* and *D. scorpiurus* exhibited significant ($p<0.05$) anti-inflammatory activities of 76.9 and 68.6% at a dose of 2000 µg/ml respectively (Figures 1 and 2).

This study shows that the anti-inflammatory activities exhibited by the leaves of *D. velutinum* and *D. scorpiurus* are mainly in the hexane fraction, and could probably be due to non-polar compounds present in the leaves. In addition, the anti-inflammatory activities exhibited by the leaves of *D. velutinum* and *D. scorpiurus* are similar to some other species of *Desmodium* previously reported: *D. gangeticum* (Govindarajan et al., 2001) and *D. triflorum* (Lai et al., 2010). The methanol extracts and fractions of *D. velutinum* and *D. scorpiurus* lowered the paw oedema from the 2nd to 6th h (Tables 6 and 7). This is similar to the effect of the methanol extract of *D. triflorum* at the 3rd to 6th h at doses of 0.5 and 1 g/kg (Shang-Chih et al., 2009). The activities of the extracts and fractions of *D. velutinum* and *D. scorpiurus* were similar to the effect of the reference drug used, (Diclofenac Sodium) which reduces inflammation by inhibiting the synthesis of prostaglandin (Della et al., 1986) through inhibition of the cyclooxygenase in the arachidonic acid pathways (Skoutakis et al., 1988; Chen et al.,1995).

The activities of flavonoids, tannins, sterols and saponins present in the leaves of *D. velutinum* and *D. scorpiurus* have been attributed to anti-inflammatory activities, and previous studies have reported the potentials of flavonoids and other phenolic compounds in exerting anti-inflammatory activities by inhibiting the enzymes involved in the mediators of inflammation (Sawadogo et al., 2006) or by inhibiting the migration of leukocyte, reducing serum lysozyme levels and nitric acid (Wu et al., 2006).

The potentials of the leaf extracts and fractions of *D. velutinum* and *D. scorpiurus* to reduce paw oedema from the second hour shows the ability of the compound(s)

Table 2. *In vivo* anti-inflammatory activities of methanol extract of *D. velutinum* leaf in egg albumin induced rats.

Dose mg/kg	0 min	1 h	2 h	3 h	4 h	5 h	6 h
50	2.93±0.07	2.90±0.06, 8.22%	2.70±0.06, 3.7%	2.63 ± 0.09, 12.3%*	2.43±0.09, 17.1%*	2.33±0.09. 17.7%*	2.27±0.07, 14.3%*
100	3.03±0.09	2.67±0.07, 15.5%	2.37 ± 0.03, 32.1%*	2.33 ± 0.03, 22.3%*	2.23 ± 0.03, 23.8%*	2.20±0.06. 22.3%*	2.10±0.06, 20.8%*
200	2.87±0.03	2.50±0.00, 20.9%	2.23 ± 0.03, 28.8%*	2.20 ± 0.00, 26.7%*	2.17 ± 0.03, 25.9%*	2.10±0.06. 25.8%*	2.07±0.03, 21.9%*
Control (Untreated)	3.37±0.07	3.16±0.06	3.13 ± 0.03	3.00 ± 0.03	2.93 ± 0.06	2.83±0.03	2.65±0.03
Diclofenac Sodium	3.10±0.06	2.93±0.15 7.3%	2.90 ± 0.16, 7.4%	2.63 ± 0.03, 12.3%	2.57 ± 0.03, 19.1%	2.37±0.09. 16.3%	2.17±0.09, 18.1%

Percentage decrease of anti-inflammatory effects compared to the untreated group,*$p<0.05$.
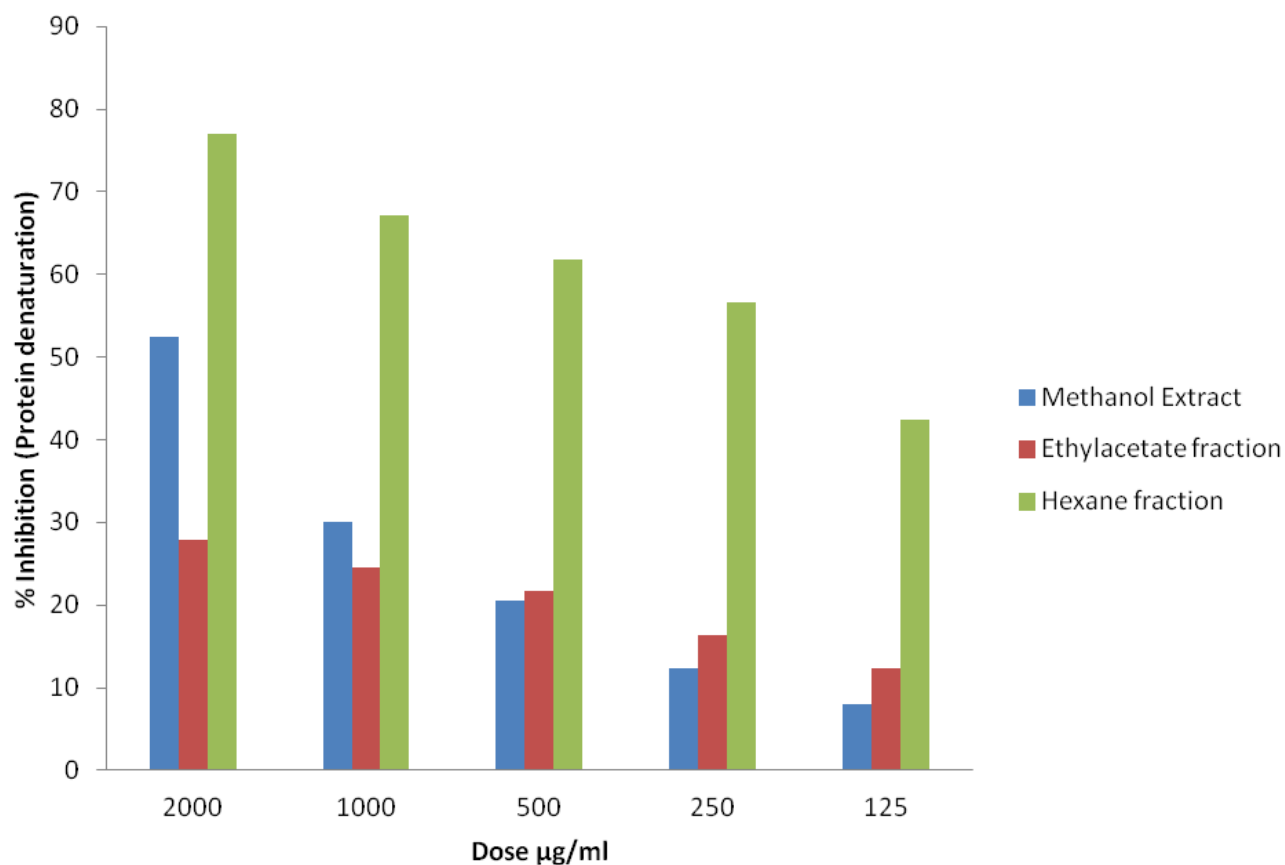
Figure 1. Effect of the methanol extract, hexane and ethyl acetate fractions of *D. velutinum* leaf against protein denaturation.

Table 3. *In vivo* anti-inflammatory activities of hexane extract of *D. velutinum* leaf in egg- albumin induced rats.

Dose mg/kg	0 min	1 h	2 h	3 h	4 h	5 h	6 h
50	3.13±0.07	3.10±0.07, 1.9%	2.90±0.06, 7.3%	2.73±0.03, 9.0%*	2.60±0.06, 11.3%*	2.40±0.09, 15.2%*	2.23±0.07, 15.8%*
100	3.00±0.06	2.77±0.03, 12.3%	2.67±0.03, 14.7%	2.47±0.03, 17.7%*	2.27±0.03, 22.5%*	2.17±0.03, 23.3%*	1.93±0.03, 27.2%*
200	3.03±0.09	2.70±0.06, 14.6%*	2.47±0.03, 21.1%*	2.40±0.06, 20.0%*	2.23±0.09, 23.9%*	2.10±0.06, 25.8%*	1.93±0.03, 27.2%*
Control (Untreated)	3.37±0.07	3.16±0.06	3.13±0.03	3.00±0.03	2.93±0.06	2.83±0.03	2.65±0.03
Diclofenac Sodium	3.10±0.06	2.93±0.15, 7.3%	2.90±0.16, 7.4%	2.63±0.03, 12.3%	2.57±0.03, 19.1%	2.37±0.09, 16.3%	2.17±0.09, 18.1%

Percentage decrease of anti-inflammatory effects compared to the untreated group, *p<0.05.
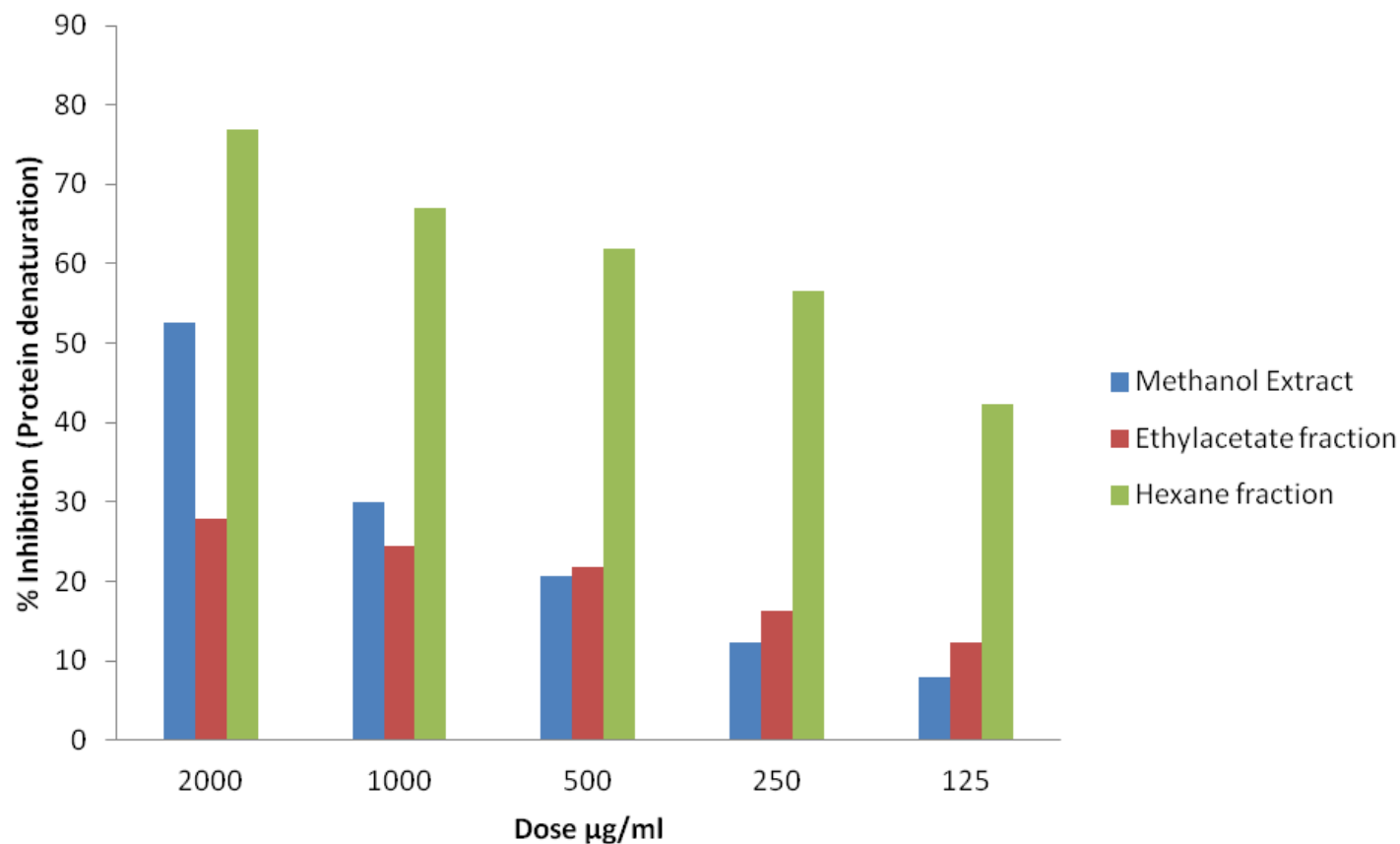
Figure 2. Effect of the methanol extract, hexane and ethyl acetate fractions of *D. scorpiurus* leaf against protein denaturation.

Table 4. *In vivo* anti-inflammatory activities of ethylacetate extract of *D. velutinum* leaf in egg- albumin induced rats.

Dose mg/kg	0 min	1 h	2 h	3 h	4 h	5 h	6 h
50	3.17±0.15	3.03±0.17,4.1%	3.03±0.17, 3.2%	2.87±0.09, 4.3%	2.83±0.12, 3.4%	2.67±0.07, 5.7%	2.57±0.07, 3.0%
100	3.30±0.06	3.03±0.08, 4.1%	2.93±0.03, 6.4%	2.83±0.03, 5.7%	2.73±0.03, 6.8%	2.53±0.06, 11.8%	2.47± 0.06, 6.8%
200	3.17±0.03	2.87±0.09, 9.2%	2.77±0.08, 11.5%*	2.63±0.03, 12.3%*	2.53±0.09, 13.7%*	2.43±0.07, 14.1%*	2.30± 0.10, 13.2%*
Control (Untreated)	3.37±0.07	3.16±0.06	3.13±0.03	3.00±0.03	2.93±0.06	2.83±0.03	2.65± 0.03
Diclofenac Sodium	3.10±0.06	2.93±0.15, 7.3%	2.90±0.16, 7.4%	2.63±0.03, 12.3%	2.57±0.03, 19.1%	2.37±0.09, 16.3%	2.17± 0.09, 18.1%

Percentage decrease of anti-inflammatory effects compared to the untreated group, *p<0.05.

Table 5. *In vivo* Anti-inflammatory activities of methanol extract of *D. scorpiurus* leaf in egg- albumin induced rats.

Dose mg/kg	0 min	1 h	2 h	3 h	4 h	5 h	6 h
50	3.10±0.15	3.07±0.19, 2.8%	2.93±0.18, 6.4%	2.67±0.13, 11.0%*	2.60±0.15, 11.3%*	2.53±0.14, 10.6%*	2.37±0.09, 10.6%*
100	3.07±0.07	2.83±0.03, 10.4%	2.80±0.06, 10.5%*	2.67±0.13, 11.0%*	2.57±0.03, 12.3%*	2.27±0.06, 19.8%*	1.93±0.07, 21.9%*
200	3.10±0.10	2.83±0.07, 10.4%	2.73±0.07, 12.8%*	2.43±0.13, 19.0%*	2.33±0.03, 20.5%*	2.23±0.07, 21.2%*	2.07±0.09, 27.1%*
Control (Untreated)	3.37±0.07	3.16±0.06	3.13±0.03	3.00±0.03	2.93±0.06	2.83±0.03	2.65±0.03
Diclofenac Sodium	3.10 ±0.06	2.93±0.15, 7.3%	2.90±0.16, 7.4%	2.63±0.03, 12.3%	2.57±0.03, 19.1%	2.37±0.09, 16.3%	2.17±0.09, 18.1%

Percentage decrease of anti-inflammatory effects compared to the untreated group, *p<0.05.

Table 6. *In vivo* anti-inflammatory activities of ethyl acetate extract of *D. scorpiurus* leaf in egg- albumin induced rats.

Dose mg/kg	0 min	1 h	2 h	3 h	4 h	5 h	6 h
50	3.07±0.07	3.07±0.07 2.8%	2.90± 0.06, 7.4%	2.87±0.07, 4.3%*	2.73±0.07, 6.8%*	2.57±0.09, 9.2%*	2.57±0.09, 3.0%*
100	2.97±0.03	2.83±0.03 10.4%	2.77±0.06, 11.5%*	2.63±0.13, 12.3%*	2.43±0.03, 17.1%*	2.30±0.06, 18.7%*	2.27±0.03, 14.3%*
200	2.97±0.03	2.73±0.03 8.1%	2.63±0.03, 15.9%*	2.50 ±0.06, 16.7%*	2.27±0.07, 22.5%*	2.20±0.00, 23.2%*	2.17±0.03, 18.1%*
Control (Untreated)	3.37±0.07	3.16±0.06	3.13±0.03	3.00±0.03	2.93±0.06	2.83±0.03	2.65±0.03
Diclofenac Sodium	3.10±0.06	2.93±0.15, 7.3%	2.90±0.16, 7.4%	2.63±0.03, 12.3%	2.57±0.03, 19.1%	2.37±0.09, 16.3%	2.17±0.09, 18.1%

Percentage decrease of anti-inflammatory effects compared to the untreated group, *p<0.05.

Table 7. *In vivo* anti-inflammatory activities of hexane extract of *D. scorpiurus* leaf in egg- albumin induced rats.

Dose mg/kg	0 min	1 h	2 h	3 h	4 h	5 h	6 h
50	2.90±0.06	2.83±0.09, 10.4%	2.80±0.10, 10.5%	2.63±0.12, 12.3%*	2.53±0.12, 13.7%*	2.43±0.12 ,14.1%*	2.40±0.12, 8.3%*
100	3.10±0.06	2.83±0.09, 10.4%	2.83±0.09, 9.6%*	2.73±0.09 9.0%*	2.67±0.09 8.9%*	2.47±0.03, 12.7%*	2.17±0.03, 18.1%*
200	2.93±0.12	2.70±0.10, 14.6%	2.67±0.09, 14.7%*	2.53±0.09, 15.7%*	2.37±0.09 19.1%*	2.17±0.03, 23.3%*	2.00±0.06, 24.5%*
Control (Untreated)	3.37±0.07	3.16±0.06	3.13±0.03	3.00±0.03	2.93±0.06	2.83±0.03	2.65±0.03
Diclofenac Sodium	3.10±0.06	2.93±0.15, 7.3%	2.90±0.16, 7.4%	2.63±0.03, 12.3%	2.57±0.03, 19.1%	2.37±0.09, 16.3%	2.17±0.09, 18.1%

Percentage decrease of anti-inflammatory effects compared to the untreated group,*p<0.05.

in the leaves to inhibit production of serotonin and bradykinnin in the first phase as well as inhibit the release of prostalglandins and nitric oxide responsible in the second phase of inflammation.

Conclusion

This study has therefore been able to further justify and report the anti-inflammatory effect of some other members of the genus Desmodium
apart from those previously studied.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antioxidant, antibacterial and cytotoxicity studies from flavonoid rich fraction of *Enicostemma axillare* (LAM.) raynal leaves

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The aim of this research was to explore the *in vitro* antioxidant, antibacterial and anticancer activities of flavonoid rich fraction from the leaves of *Enicostemma axillare*. The total phenolic and flavonoid contents were assessed by Folin–Ciocalteu and Aluminium chloride method respectively. Antioxidant activities of this plant was confirmed on the source of ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid), Radical scavenging assay, Inhibition of lipid peroxidation, Super oxide radical scavenging activity, Nitric oxide radical scavenging activity and Metal chelating activity. Correspondingly, antibacterial activities were accomplished by disc diffusion method and MIC (Minimum Inhibitory Concentration) against *Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pnemoniae, Escherichia coli* and *Enterococcus faecalis* and anticancer activity was performed against human breast cancer cell MCF-7. The total phenolic and flavonoid content in flavonoid rich fraction of *E. axillare* was 136.8±13.00 mg GAE/g and 75.2±1.23 µg RE/g respectively. The flavonoid rich fraction of the leaves of *E. axillare* exhibited significant antioxidant activity. Similarly, the study on antibacterial activity of flavonoid rich fraction of *E. axillare* exposed inhibitory activity. However flavonoid rich fraction of *E. axillare* showed high inhibitory zone against *E. coli, S. aureus, K. pneumoniae* (18, 15 and 14 mm). The flavonoid rich fraction of *E. axillare* also unveiled strong cytotoxic effect with IC_{50} values of 15.39 µg/ml against MCF-7. This research work has made it clear that *E. axillare* possess excellent antioxidant, antibacterial and cytotoxic activity and the extracts can be more extensively used in developing countries for the prevention and treatment of ageing and infective associated diseases and may be considered as good source for drug discovery.

Key words: *E. axillare,* flavonoid rich fraction, antioxidant, antibacterial, cytotoxic activity.

INTRODUCTION

Plants have been used in ancient times as medicine to treat several diseases and medical complaints by most, if not all civilizations. Herbal-therapy in India is predominantly extensive and documented. Similarly plant

based medicinal system remain to be the primary therapeutic system in various parts of India. Recently, consumer's aspect to decrease the risk or accomplish a specific health ailment through improved food diet. Plants and fruits have diverse phytochemicals and enzymes as antioxidant defense to sustain growth and metabolism system (Pandhair and Sekhon, 2006). The research on antioxidant activity has been improved due to the apprehension about health enhancement involving agricultural products with their probable benefits (Moore et al., 2005). Antioxidants that are chiefly supplied as dietary ingestions can obstruct carcinogenesis by scavenging free radicals or interfering with binding of carcinogens to deoxyribonucleic acid. Several reports have exposed that the majority of the antioxidant activity may be from phytochemicals such as flavonoids, isoflavones, flavones, anthocyanins, catechins and other phenolics (Alothman et al., 2009; Isabelle et al., 2010).The antioxidative abilities of phenolic compounds can be accredited to their strong capacity to transfer electrons to reactive oxygen species or free radicals, chelating metal ions by stimulating antioxidant enzymes and inhibitory oxidases (Choi and Lee, 2009). In addition, free radicals and reactive oxygen species are continuously produced in-vivo and cause oxidative injury to biomolecules, a process held to check only by the presence of multiple antioxidants or repair systems as well as the replacement of injured lipids or proteins (Bapjpai et al., 2009).

As a consequence, many irredeemable human diseases including cancer, cardio and cerebro-vascular diseases have been recognized (Hossain et al., 2015). The possible ways to fight these irredeemable diseases is to progress our body's transformation due to antioxidant defenses. High ingestion of plants, fruits and vegetables has lowered the incidence of such deteriorating or irredeemable diseases (Hossain et al., 2006). Plants can contribute in this area chiefly due to the antioxidant activity of polyphenolic compounds (Feng et al., 2006). Flavonoids that mainly exists as colouring pigments in plants too function as potent antioxidants at innumerable levels. Some reports exhibited that flavonoids could shield membrane lipids from oxidation (Cos et al., 1998). Microbial contamination is one of the major cause responsible to induce oxidative reactions which intern lead to cell damage (Bhargava et al., 2010; Cabiscol et al., 2000). Although many antimicrobials have been effectively used but remarkable flexibility and the development of resistance are major problems (Grant and Hung, 2013). Similarly, Cancer is also one of the world's shocking diseases leading to disease-related impermanence and anomalous growth of cells and tissues. Its treatment includes surgical, radiation,

chemotherapeutic drugs which often destroys healthy cells and cause toxicity to humans. Drug designs for cancers are well evolving due to the overview of plant molecules. Among this, breast cancer is one of the most common cancer and the prominent cause of cancer-associated deaths in females (Sorlie et al., 2001). It has been categorized into several subgroups based on the pathology and gene expression profiles, which subsidize to several responses to the same therapeutic strategies (Stockler et al., 2000).

Enicostemma axillare (Lam.) Raynal (Syn *Enicostemma littorale* Blume, Gentianaceae) is a perennial herb widely distributed all over India and common in coastal areas. According to Ethnobotanical information this plant is used to treat diabetes mellitus, rheumatism, abdominal ulcers, hernia, swelling, itching and insect poisoning. In pharmacological research, it's been reported that this plant possess anti-inflammatory, hypoglycemic and anticancer activities (Roy et al., 2010). Swertiamarin, alkaloids, steroids, triterpenoids, saponins, flavonoids, xanthones, phenolic acids, etc. were isolated from the *E. axillare* (Ghosal et al., 1974). Keeping this in view, there has been considerable interest by the industry and a growing trend in consumer preferences for natural antioxidants and antimicrobial compounds and elimination of synthetics food applications has given more impetus to explore natural sources. Up to date, the search for new natural antioxidant, antimicrobial and anticancer agents from plants remain a potential area of investigation. Thus, they are of interest to both food and health scientists and there has been a convergence of interest among researchers in these fields and their impact on human health has come under attention. The study is therefore designed to determine antioxidant, antimicrobial and anticancer activity of flavonoid rich fractions from the leaves of *E. axillare*.

MATERIALS AND METHODS

Plant materials

Leaves of *E. axillare* were collected from Government Siddha Medical College, Herbal Garden, Chennai 600 106, Tami Nadu, India. Plant was authenticated by Dr. S. Sankaranarayanan, Head, Department of Medicinal Botany, Government Siddha Medical College, Arumbakkam, Chennai-600 106, Tamilnadu.

Extraction of flavonoid rich fraction

The Leaves of *E. axillare* were dried in hot air oven at (40°C) for 1 h, after which it was pulverized to uniform powder with house hold mixer grinder. The aqueous extract was prepared by soaking 100 g

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of the dry powdered plant materials in 500 ml of aqueous at 4°C for 24 h. The extract was filtered first through a What mann filter paper No. 42 (125 mm) and then centrifuged at 5000 rpm for 10 min (Remi-R-8C, India). The clear solution was separated with petroleum ether and chloroform for exclusion of other than flavonoid compound and finally aqueous extracts were partitioned with butanol. It was condensed using a rotary evaporator with the water bath set at 40°C. The percentage yield of extracts extended from 7 to 19% w/w.

Determination of total phenolics

The concentration of total phenolics in the flavonoid extract of *E. axillare* was resolute by using Folin-Ciocalteu reagent and standardized externally with gallic acid. Briefly, 0.2 ml of flavonoid rich fraction and 0.2 ml of Folin-Ciocalteu reagent were added and mixed strongly. After shaking for 4 minutes, 1 ml of 15% Na_2CO_3 was added, and finally the combination was allowed to stand for 2 h at room temperature. The absorbance was measured at 760 nm using Deep Vision 1371 spectrophotometer. The concentration of the total phenolic was assessed as mg of gallic acid comparable by using an equation obtained from gallic acid calibration curve. The quantification of phenolic content was carried out in triplicate and the results were averaged (Singleton et al., 1999).

Determination of flavonoid content

The amount of total flavonoids in the extract was measured according to Quettier-Deleu et al. (2000). This method is based on the formation of a complex flavonoid-Aluminium, with the absorbance maximum at 430 nm. Rutin was used to brand a calibration curve. To 1 ml of flavonoid rich fraction, added 1 ml of 2% $AlCl_3$ and it was incubated at room temperature for 15 min. Then absorbance was measured at 430 nm using Deep Vision 1371 spectrophotometer.

Invitro antioxidants properties

ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical scavenging assay

ABTS radical scavenging activity of flavonoid rich fraction of *E. axillare* was followed by Re et al. (1999). ABTS radical was newly prepared by addition 5 ml of 4.9 mM potassium persulfate solution to 5 ml of 14 mM ABTS solution and kept for 16 h in dark. This solution was diluted with distilled water to produce an absorbance of 0.70 at 734 nm and the same was used for the antioxidant activity. The final solution of standard group was made up to 1 ml with 950 µl of ABTS solution and 50 µl of Ascorbic acid. Correspondingly, in the experiment group, 1 ml reaction mixture encompassed 950 µl of ABTS solution and 50 µl of different concentration of flavonoid rich fraction. The reaction mixture was vortexed for 10 s and after 6 min, absorbance was recorded at 734 nm against distilled water by using a Deep Vision (1371) UV–Vis Spectrophotometer and compared with the control ABTS solution. Ascorbic acid was used as reference antioxidant compound. ABTS Scavenging Effect (%) = [(A_0- A_1/A_0) ×100] Where $A0$ is the absorbance of the control reaction and $A1$ is the absorbance of flavonoid rich fraction.

Inhibition of lipid peroxidation activity

Lipid peroxidation induced by Fe^{2+} ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS)

by the method of Ohkawa et al. (1979). The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of flavonoid rich fraction of *E. axillare* in a final volume of 0.5 ml. The experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard.

Inhibition of lipid peroxidation (%) by the flavonoid rich fraction was calculated according to 1-(E/C) × 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample (Abs_{532}+TBA–Abs_{532}+TBA).

Superoxide radical scavenging assay

This assay was based on the capacity of the flavonoid rich fraction to inhibit the photochemical reduction of Nitroblue tetrazolium (NBT) in the presence of the riboflavin-light-NBT system (Tripathi and Pandey Ekta, 1999; Tripathi and Sharma, 1999). Each 3 ml reaction solution contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 µM riboflavin, 100 µM Ethylene diamine tetra acetic acid (EDTA), NBT (75 µM) and different concentration of flavonoid rich fraction. It was kept visible in fluorescent light and absorbance was taken after 6 min at 560 nm by using a Deep Vision (1371) UV–Vis Spectrophotometer. Identical tubes with reaction mixture were kept in the dark served as blanks. The percentage inhibition of superoxide radical activity was measured by comparing the absorbance of the control with test sample solution:

% Super oxide radical scavenging capacity= [(A_0-A_1)/A_0] ×100
Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

Nitric oxide radical scavenging activity

Nitric oxide scavenging ability of flavonoid rich fraction of *E. axillare* was measured according to the method described by Olabinri et al. (2010). 0.1 ml of sodium nitroprusside (10 mM) in phosphate buffer (0.2 M, pH 7.8) was mixed with different concentration of flavonoid rich fraction and incubated at room temperature for 150 min. After treated period, 0.2 ml of Griess reagent (1% Sulfanilamide, 2% Phosphoric acid and 0.1% N-(1-Naphthyl) ethylene diamine dihydrochloride) was added. The absorbance of the experimental sample was read at 546 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The percentage of inhibition was calculated by following equation:

% Nitric oxide radical scavenging capacity= [(A_0-A_1)/A_0] ×100

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

Metal chelating activity

Metal chelating capacity of flavonoid rich fraction of *E. axillare* was

measured according to Iihami et al., (2003). 1 ml of different concentrations of flavonoid rich fraction was added to 0.05 ml of 2 mM ferric chloride solution. The reaction was initiated by the addition of 0.2 ml of 5 mM Ferrozine and the mixture was shaken vigorously. After 10 min, the absorbance was measured at 562 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The % inhibition of ferrozoine-Fe2+complex was calculated by following equation.

% Inhibition of ferrozoine-Fe^{2+}complex = [(A$_0$-A$_1$)/A$_0$] ×100

Where A$_0$ was the absorbance of control and A$_1$ was the absorbance of flavonoid rich fraction.

GC–MS analysis

The flavonoid fraction was analyzed by GC–MS (QP-2010, Shimadzu Co., Kyoto, Japan) equipped with 30- mX 0.25 mm DB-5MS column (Agilent Technologies, J&W Scientific Products, Folsom, CA). The carrier gas was helium. The temperature program was set as follows: 100°C hold for 5 min, raised at 4°C/min to 280°C, and hold for 5 min. The injector and detector temperatures were set at 250 and 280°C, respectively. The ion source and interface temperatures were set at 200 and 250°C, correspondingly. The mass range was scanned from 50 to 900 amu. The control of the GC–MS system and the data peak processing were controlled by Shimadzu's GC–MS solution software, version 2.4. Compound identification was confirmed based on the relative retention time and mass fragmentation pattern spectra with those of standards and the NIST147. LIB. Database of the GC–MS system (Shimadzu).

Antibacterial properties

Bacterial strains

Bacteria used for the assessing antibacterial activities were Gram positive viz; *Staphylococcus aureus* MTCC 29213, *Klebsiella pneumoniae* MTCC 1771 and *Enterococcus faecalis* MTCC 439 and gram negative viz; *Pseudomonas aeruginosa* MTCC 2488, and *Escherichia coli* MTCC 25922. The bacterial strains were obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology Sector 39-A, Chandigarh-160036, India. All bacterial strains were sub cultured on nutrient agar medium, incubated at 37°C for 24 h and stored at 4°C in refrigerator to maintain stock culture.

Antibacterial assay

Antibacterial activity was assessed using disc diffusion method (Velickovic et al., 2003). Petriplates were prepared with 20 ml of sterile nutrient agar (HIMEDIA). The tested cultures were swabbed on top of the solidified media and allowed to dry for 10 min. The test was conducted in four different concentrations of the flavonoid rich fraction (5, 10, 15 and 20 µl/ml) and treated discs (Whatman No.1 filter paper was used to prepare discs) were air dried. The treated discs were placed on the surface of the medium and incubated at room temperature for 24 h. The relative inhibition of organisms to the flavonoid rich fraction was indicated by a clear zone of inhibition around the discs. It was then detected, measured and documented in millimeters with three replicates.

Cytotoxicity screening

The cytotoxicity of flavonoid rich fraction of *E. axillare* was

determined by Methyl tetrazolium (MTT) assay (Selvakumaran et al., 2003). Cells (2×10^3/well) were placed in 100 µl of medium/well in 96-well plates. After overnight incubation, flavonoid rich fraction was added in 5 wells for each concentration. After treating with flavonoid rich fraction for 1st, 2nd, 3rd, 4th, and 5th day, 20 µl of 5 mg/ml MTT (pH 4.7) was added to each well and cultured for another 4 h. The supernatant was eliminated and 100 µl DMSO was added per well. After shaking the samples for 15 min. the absorbance was measured at 570 nm with microplate reader (Bio-Rad), using wells without cells as blanks. All experiments were performed in triplicate. The treated cell line was expressed relatively on its cell viability, using the following formula.

$$\text{Percent viability} = \frac{\text{OD of drug treated sample}}{\text{OD of none treated sample}} \times 100$$

Statistical analysis

The impact of the flavonoid rich fraction of *E. axillare* on its antioxidant activity was measured by the ABTS assay, lipid peroxidation, superoxide scavenging, metal chelating and nitric oxide radical were determined using one-way analysis of variance (ANOVA). Likewise, Duncan's post hoc test was applied, so as to determine the statistically significant different values. All statistical handling was performed using SPSS software, v. 14.0 (SPSS, Chicago, Ill., U. S. A.).

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

E. axillare exhibited the highest amount of total phenolic (172.08±13.00 mg/g) and flavonoid contents (75.2±1.23 µg RE/g). Flavonoids has extensive health benefits, as anti-allergic, anti-inflammatory, analgesic, and cardio protective, hepatoprotective, anticancer, antiviral activities. These diverse properties of Flavonoids are due to its varied structures (Agrawal, 2011). The above data in accordance with previous researches (Maisuthisakul et al., 2007), had revealed that the high total polyphenols content increases antioxidant activity and there was a linear correlation between phenolic and flavonoid content for antioxidant activity.

Free radical-scavenging ability using ABTS assay

The radical scavenging ability was measured by ABTS assay as per given in table 2. The inhibition percentage of the ABTS radical activity was assessed on average and high free radical-scavenging values were found in flavonoid rich fraction of *E. axillare*. In ABTS assay, the EC$_{50}$ of the pure ascorbic acid (29.97 µg/ml) was lower than *E. axillare*, (18.97 µg/ml) (Table 1). Nevertheless, in our study, it is showed that these activities were mainly due to phenolics and flavonoids. It is known that vitamin C (ascorbic acid) and carotenoids are chief source of discrepancy of antioxidant/ antiradical activities in plant

Table 1. Free radical-scavenging ability using ABTS assay.

Flavonoid rich fraction and Positive control	[a]ABTS radical Scavenging			
	5 µg/ml	10 µg/ml	15 µg/ml	20 µg/ml
E. axillare	31.35±0.55[*]	53.65±0.61[*]	69.04±0.55[*]	87.36±0.55[**]
Ascorbic acid	14.92±0.75	29.89±0.69	45.00±0.48	60.89±0.75

[a]Values are means of three replicates ± standard deviation. Flavonoid rich fraction of *E. axillare*; Means with superscripts having two asterisk in the same column are significantly ($p < 0.05$) different.

Table 2. Inhibition of lipid peroxidation by Flavonoid rich fraction of *E. axillare*.

Flavonoid rich fraction and Positive control	Inhibition of Lipid peroxidation			
	5 µg/ml	10 µg/ml	15 µg/ml	20 µg/ml
E. axillare	28.26±1.25[*]	49.7±1.15[**]	62.10±1.17[*]	81.93±0.94[*]
Ascorbic acid	11.33±1.52	25.33±1.52	40.33±3.51	50.66±2.08

[a]Values are means of three replicates ± standard deviation. Flavonoid rich fraction of *E. axillare*; Means with superscripts having two asterisk in the same column are significantly ($p < 0.05$) different.

Table 3. Superoxide scavenging assay activity by Flavonoid rich fraction of *E. axillare*

Flavonoid rich fraction and Positive control	Superoxide scavenging assay			
	5 µg/ml	10 µg/ml	15 µg/ml	20 µg/ml
E. axillare	27.11±1.17[*]	47.70±1.17[*]	65.49±0.93[**]	78.31±1.29[*]
Ascorbic acid	11.18±0.84	24.16±1.40	41.79±0.58	57.49±0.71

[a]Values are means of three replicates ± standard deviation. Flavonoid rich fraction of *E. axillare*; Means with superscripts having two asterisk in the same column are significantly ($p < 0.05$) different.

materials. Although these components were not explored, their contributions towards antioxidant/ antiradical activities of these studied medicinal plants may be insignificant. The presence of phenolic compounds in plants shows a synergic effect of antioxidants in association with phytochemicals (Kumar and Pandey, 2013; Teow et al., 2007).

Inhibition of lipid peroxidation

Flavonoid rich fraction of *E. axillare* also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was recorded in flavonoid rich fraction of *E. axillare* with EC_{50} value at 9.28 µg/ml and ascorbic acid 32.98 µg/ml. As it is identified that lipid peroxidation is the net result of any free radical attack on membrane and other lipid components present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Significantly, *E. axillare* suppressed the degree of lipid

peroxidation than positive control (Table 2). Normally, the mechanism of phenolic compounds for antioxidant activity includes neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals (Prior et al., 2005). In previous studies it is recommended that the Pasteurization of blackberry juice preserves polyphenol-dependent inhibition for lipid peroxidation and intracellular radical (Kim et al., 2002; Gabriela et al., 2015).

Superoxide scavenging assay activity

Flavonoid rich fraction of *E. axillare* exhibited powerful scavenging activity for superoxide radicals in a concentration dependent process than positive control. Flavonoid rich fraction of *E. axillare* showed EC_{50} values of 19.39 µg/ml and the positive control with an EC_{50} value of 20.50 µg/ml (Table 3). One of the standard method to produce Superoxide radicals is through photochemical reduction of nitro blue tetrazolium (NBT) in the presence of a riboflavin-light-NBT system. These superoxide radicals are extremely toxic and may be produced either through xanthine activity or through mitochondrial

Table 4. Nitric oxide radical scavenging assay by Flavonoid rich fraction of *E. axillare*.

Flavonoid rich fraction and positive control	[a]Nitric oxide radical scavenging			
	5 µg/ml	10 µg/ml	15 µg/ml	20 µg/ml
E. axillare	29.53±0.91[**]	49.72±0.81[**]	66.46±0.81[*]	83.26±0.71[*]
Ascorbic acid	10.97±0.53	26.28±0.91	42.61±0.50	61.51±0.71

[a]Values are means of three replicates ± standard deviation. Flavonoid rich fraction of *E. axillare*; Means with superscripts having two asterisk in the same column are significantly ($p < 0.05$) different.

Table 5. Metal chelating activity Flavonoid rich fraction of *E. axillare.*

Flavonoidrichfraction and Positive control	[a] Metal chelating activity			
	5 µg/ml	10 µg/ml	15 µg/ml	20 µg/ml
E. axillare	25.90±1.67[**]	46.22±1.64[*]	58.75±1.67[*]	78.70±3.44[*]
Ascorbic acid	11.91±0.92	23.71±1.31	38.31±1.09	51.09±1.46

[a]Values are means of three replicates ± standard deviation. Flavonoid rich fraction of *E. axillare*; Means with superscripts having two asterisk in the same column are significantly ($p < 0.05$) different.

reaction. Superoxide radicals are reasonably a weak oxidant may decompose to form stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals (Dirk et al., 2003). The present study recommended that *E. axillare* possessed strong scavenging activity for superoxide radicals in a concentration dependent process than positive control. The presence of secondary metabolites like Flavonoids, carotenoids and triterpenes in higher plants have excellent antioxidant activity by scavenging reactive oxygen species which prevent possible damage to cellular components such as DNA, proteins and lipids (Ksouri et al., 2013).

Nitric oxide radical scavenging assay

Nitric oxide radical quenching activity of the Flavonoid rich fraction of *E. axillare* was identified and compared with the standard ascorbic acid. The flavonoid rich fraction of *E. axillare* displayed the maximum inhibition of 68% at a concentration of 20 µg/ml with an EC_{50} value of 29.42 µg/ml, in a concentration-dependent process when compared to ascorbic acid with EC_{50} value of 59.48 µg/ml (Table 4). In the current study, nitrite was produced by incubation of sodium nitroprusside in standard phosphate saline buffer at 25°C was reduced by flavonoid rich fraction. Significant scavenging activity may be due to the antioxidant property of flavonoid, which compete with oxygen to react with nitric oxide, leading to less production of nitric oxide (Alasalvar et al., 2006).

Metal chelating activity

The metal chelating property of Flavonoid rich fraction of

E. axillare was displayed as per Table 5. The Flavonoid rich fraction of *E. axillare* was evaluated for their ability to compete with ferrozine for ferrous iron in the solution. In this evaluation, the leaf extract of *E. axillare* hindered the formation of ferrous and ferrozine complex, signifying that they have chelating activity and are capable of capturing ferrous iron before ferrozine. The Flavonoid rich fraction of *E. axillare* reduced the red color complex immediately and showed the highest chelating activity (EC_{50}7.59 µg/ml) than positive control (EC_{50}27.11 µg/ml). It's concluded that flavonoid compounds in extract would be responsible for antioxidant activities and reducing ferric ions might not be directly involved in ferrous ion chelation. This result was in accordance with the data that chelating agents, that form σ-bonds with a metal, are effective as secondary antioxidants as they reduce the redox potential, there by stabilizing the oxidized form of the metal ion (Kumaran and Karunakaran, 2006).

Anti-bacterial activity of Flavonoid rich fraction of *E. axillare* tested against pathogenic bacteria

Antibacterial activities of the Flavonoid rich fraction of *E. axillare* was tested against pathogenic organisms as per Table 6. The plants vary in their activities against the micro-organisms tested. Flavonoid rich fraction of *E. axillare* exhibited maximum antibacterial activity against *Klebsiella pneumoniae, Escherichia coli, Enterococcus faecalis, Staphylococcus aureus* than *Pseudomonas aeruginosa.* Highest antibacterial activity was recorded with flavonoid rich fraction of *E. axillare* against *E. coli, S. aureus, K. pneumoniae* (18, 15 and 14 mm) respectively while lowest activity was recorded against *E. faecalis,* and *P. aeruginosa* with the inhibition zone of 13.9 and 13.4 mm. The current investigation revealed that

Table 6. Anti-bacterial activity of Flavonoid rich fraction of *E. axillare* tested against pathogenic bacteria.

Bacteria	[a]Inhibition zone measured (mm)				
	5 µl/ml	10 µl/ml	15 µl/ml	20 µl/ml	Positive control Streptomycin 10 mcg
Klebsiella pneumoniae	7.46±0.30	9.8±0.36	12.9±0.2	14.9±0.3	9.5±0.5
Enterococcus faecalis	7.6±0.55	9.8±0.55	12.1±0.2	13.8±0.45	10.2±0.3
Staphylococcus aureus	8.66±0.77	10.5±1.11	12.7±0.6	15.9±0.79	9.8±07
Escherichia coli	9.2±0.65	11.3±0.35	14.83±0.65	18.2±0.55	10.2±0.6
Pseudomonas aeruginosa	7.3±0.25	9.7±0.30	11.63±0.30	13.46±0.41	10.8±0.3

[a] Mean diameter of the zone of inhibition is in millimeters.
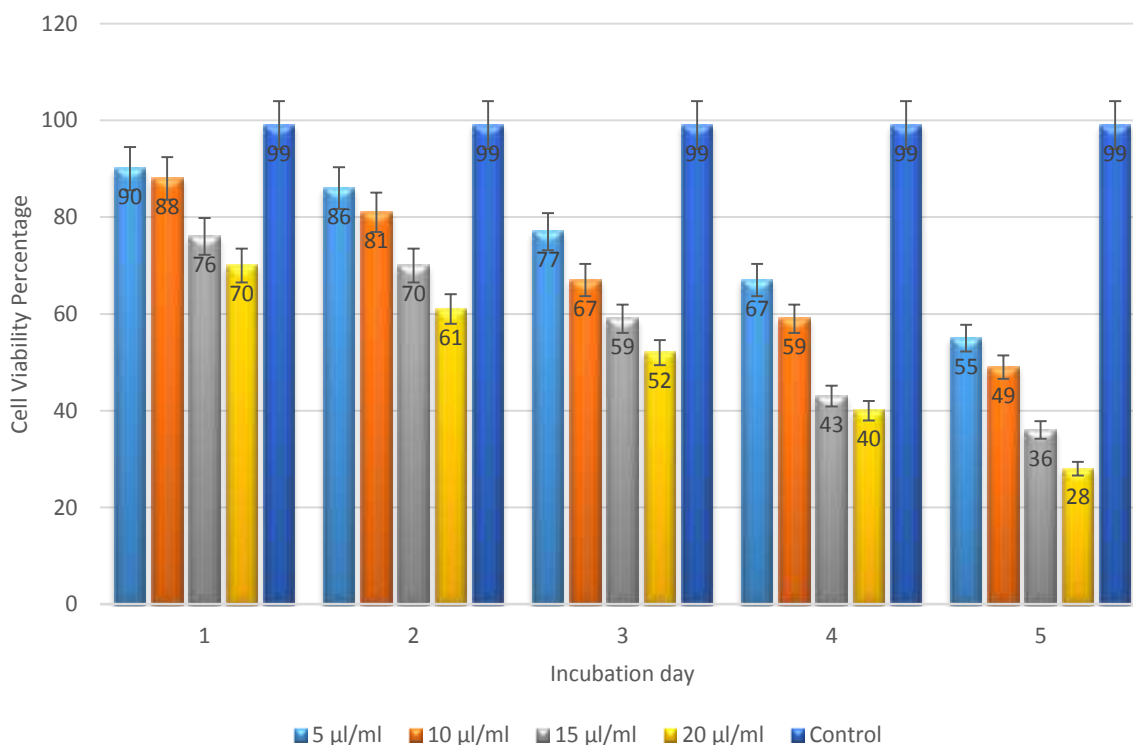

Figure 1. Cytotoxicity of Flavonoid rich fraction of *E. axillare* on MCF-7 cancer cell. All data are expressed as the mean ± SE of three experiments.

Flavonoid rich fraction has potential antibacterial activity against entire tested organisms. Similar investigation was reported in methanol extract exhibiting strongest and broadest spectrum of anti-bacterial activity (Chan et al., 2007). Previous research reveals that plant extracts are less active in Gram Negative bacteria than Gram Positive bacteria as it has an outer membrane consisting of lipoprotein and lipopolysaccharide, which is selectively permeable and thus regulates access to the underlying structures (Ghadir et al., 2014).

Effects of cytotoxicity on human breast cancer cell MCF-7

Cytotoxic effects of Flavonoid rich fraction of *E. axillare* was observed by using MTT assay against human breast cancer cell lines MCF-7 for daily treatment of 24, 48, 72, 96 and 120h, respectively. As shown in Figure 1, Flavonoid rich fraction of *E. axillare* displayed strong cytotoxic effect with IC_{50} values of 15.39 µg/ml against MCF-7. Cell morphological variations too indicated that

Table 7. Analysis of flavonoid rich fractions by GC–MS.

S/N	Compound	Retention Time(min)	Molecular weight	Major peaks
1	Catechin	15.16	290	113.1, 99, 85.01
2	Phenol, 2,6-dimethoxy-	16.18	154	154, 140, 130, 126
3	Decane, 2,3,5,8-tetramethyl-	16.86	198	155, 151, 141, 127
4	4-Propylphenol	17.18	136	136, 121, 107, 99
5	Benzaldehyde, 2-hydroxy-3-methoxy-	17.88	152	152, 122, 106, 81
6	*3-Hydroxy-4-methoxybenzoic acid.*	18.97	168	168, 153, 125, 97
7	3,4-dihydroxybenzoic acid; Protocatechuic acid	18.1	154	154, 137, 109, 81
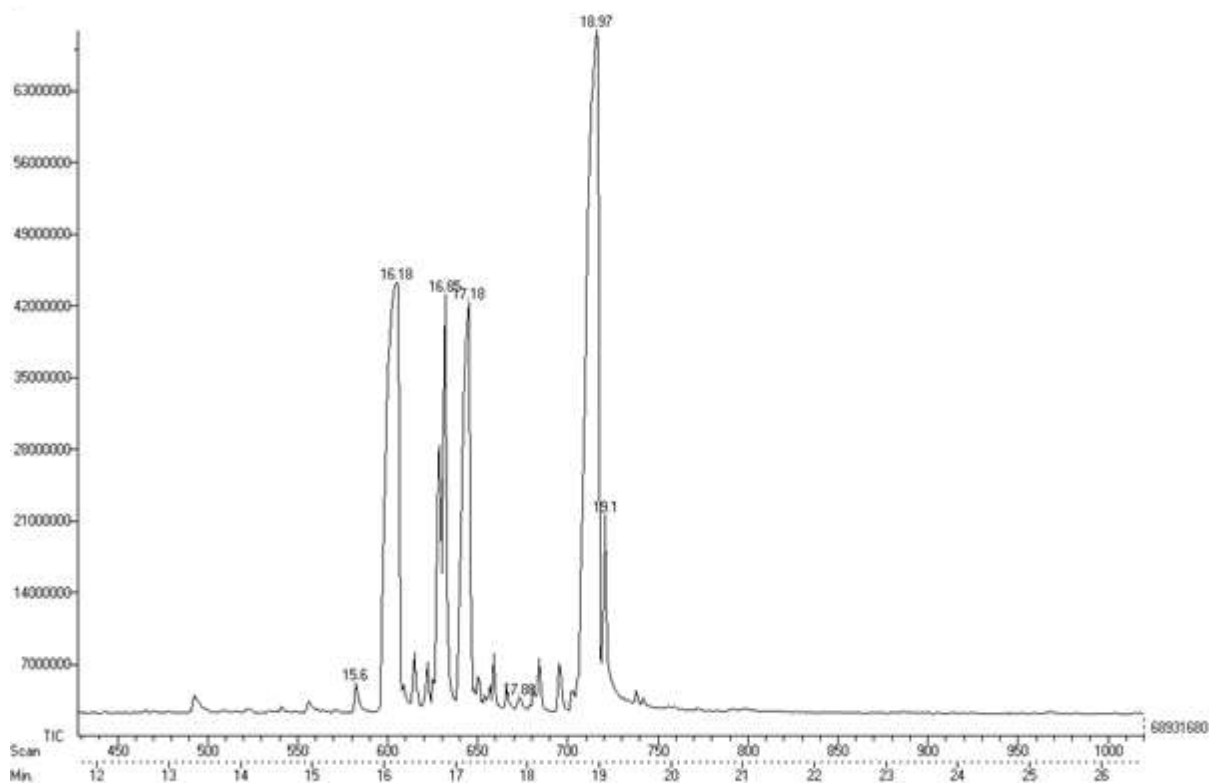
Figure 2. Analysis of flavonoid rich fraction of *E. axillare* By GC-MS.

the treatment of breast cancer cells at various doses of flavonoid rich fraction reflected its significant cytotoxicity to human breast cancer cells at doses of 5 µg/ml and above with high cytotoxic effect. Current investigation recommended that the flavonoid rich fraction of *E. axillare*, with a high phenolic content, possesses stronger anticancer activity. Natural compounds copious in plants are capable of arresting cell growth in tumor cells and modulating cell signaling pathways associated to cell death (Zou and Chang, 2011). The present study was in accordance with the previous findings which revealed that poly-phenolic extracts from several plants exhibited high efficiency in antitumor activity (Pan et al., 2008).

Analysis of flavonoid rich fractions by GC–MS

The flavonoid rich fraction of the *E. axillare* ensured highest flavonoid content and exhibited the strongest antioxidant activity (Table 7). It was therefore analyzed by GC–MS to determine its chemical composition that may contribute to this activity. The GC–MS analysis showed a variety of phenolic compounds (Figure 2).

Conclusion

The flavonoid rich fraction of *E. axillare* can protect the

body from oxidative stress may be due to the existence of phytochemicals in the form of flavonoids compounds. These phytochemicals are present in this plant and it contributes to their medicinal properties that can be used in nutraceuticals and pharmaceutical industry. Nevertheless, additional studies are essential to develop a technique for the fractionation and identification of most active antioxidant, antibacterial and anticancer molecules and thus can be used in the prevention and treatment of ageing and infective related diseases and can be considered as good source for drug discovery.

Conflict of Interests

The authors have not declared any conflict of interests.

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Full Length Research Paper

Development, characterization and assessment of botulinum toxin type A incorporated in nanocarriers

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Botulinum toxin type A is one of the most used products in mitigation of wrinkles and expression lines by promoting muscle paralysis. Because of their hydrophilic and high molecular mass, this active has low permeability through the skin. Thus, to exert its action as rejuvenation agent, botulinum toxin needs to be applied through intramuscular injections, causing pain and discomfort to the patient. An alternative to minimize this problem is encapsulation of the neuropeptide in nanocarriers that can easily cross the skin barrier. Thus, the aim of this work was to study the feasibility of encapsulation of botulinum toxin A in polymeric nanoparticles and liposomes. It was possible to demonstrate the formation of nanoparticles and liposomes loaded with botulinum toxin A. It was observed that liposomes containing botulinum toxin had an effect on neurotransmitters most effective in tests *in vitro*. The results demonstrate that it is possible to encapsulate botulinum toxin type A in liposome form without altering its biological action.

Key words: Botulinum toxin type A, liposomes, nanoparticles.

INTRODUCTION

Wrinkles are the most expressive signs of cutaneous aging, which is a complex biological phenomenon that depends on a combination of intrinsic and extrinsic factors. The intrinsic factors are responsible for chronological aging that is genetically determined and one of the main extrinsic factors is exposure to solar radiation that leads to photo-aging and causes alterations in the skin such as wrinkles, dry skin, loss of elasticity, irregular pigmentation, atrophy, collagen fragmentation and elastic fibers. The accumulation of elastin below the dermal-epidermal junction characterizes these effects and the elastosis that is typical of this aging (Khavkin and Ellis 2011). In addition, other factors must be considered, such as repetitive facial movements caused by a contraction of the facial muscles (Buck et al, 2009) that can lead to wrinkles or signs of expression. Since over a

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period of time, muscles become hypertrophied and acquire an increased tonicity. This natural phenomenon means that the furrows and wrinkles remain apparent even when the muscle is not contracted and the skin becomes flaccid and the wrinkles accentuated into deep grooves (Batistela et al, 2007).

Currently, the search for anti-aging products that can repair this phenomena, suggest the use of botulinum toxin type A, which is the regarded method for wrinkles. It is naturally produced from *Clostridium botulinum*, an anaerobic bacterium that produces seven serological types of toxin and type A is used clinically because it is the most powerful (Sposito 2004). Botulinum toxin acts by preventing the release of acetylcholine in the neuromuscular junction and causes paralysis (Blanes-Mira et al, 2002). In addition, this prevention is involved with the process of insulin release, that is, if there is no release of acetylcholine, there will be no release of insulin. Acetylcholine, released from nerve endings of the vagus nerve (parasympathetic nervous system), cholecystokinin, released by enteroendocrine cells from the intestinal mucosa, and the gastrointestinal inhibitory peptide are some substances that stimulate the release of insulin Kiba (2004). This neurotoxin was approved in 1989 for use in the treatment of squinting, eye-twitching and hemifacial spasms. In 2002, it was approved for cosmetic use to mitigate facial lines and in 2004 for the treatment of hyperhidrosis Mahajan and Brubaker (2007).

Botulinum toxin type A is a hydrophilic neuropeptide with high molar mass. It has a low permeability through the skin, mainly because of the lipophilic characteristics of the stratum corneum Gorouhi and Maibach (2009). Therefore, to carry out rejuvenation, botulinum toxin has to be applied by intramuscular injection; however, this procedure causes pain and discomfort to the patient.

Regarding this problem, technological development of new pharmaceutical methods has proved to be a more promising strategy to allow a free passage of medicines through the skin. There has been a great interest in the selective release of medication in the skin and hence there have been several studies of carrier systems with the aim of improving the selection and efficiency of the formulations Bruschi (2010).
Nanoparticles and liposomes are the most important of these carriers. Liposomes are spherical vesicles formed by lipid bilayers with a size that can vary from 20 nm to a number of micrometers. They can be used to encapsulate hydrophilic drugs in the aqueous cavity of the vesicle or lipophilic drugs in the lipid bilayer Lasic (1998) and Tyagi et al. (2006).

According to the process and composition of the nanoparticles, it is possible to obtain nanospheres or nanocapsules. Regarding the nanocapsules, the drug is found in a nucleus of the particle and nanospheres. The drug is encapsulated or adsorbed in the polymer mesh Brigger et al, (2002). The size of the nanoparticles varies

from 10 to 1000 nm Pimentel et al. (2007).

In order to alleviating the discomfort of the patient, it is possible to encapsulate botulinum toxin into liposomes or even into nanoparticles. This can carry out their topical application and ensure their penetration and activity in the skin. There were many reports in the literature about the encapsulation of botulinum toxin Type A which underlines the innovative nature of this study.

MATERIALS AND METHODS

Reagents and solvents

Onabotulinumoxin A (Botox® 100 U, Allergan Pharmaceuticals, Westport, Irlanda); Poly(vinyl alcohol) (PVA) (*Mw* 13.000 a 23. 000 g.mol^{-1}, Sigma-Aldrich, St. Louis, USA); Cholesterol (Sigma-Aldrich, St. Louis, USA); Phosphatidylcholine (EMBRAFARMA, São Paulo, Brazil); Poly (*ε*-caprolactone) (PCL) (Mw70.000 a 90.000 g.mol^{-1}, Sigma-Aldrich, St. Louis, USA); and chemicals with analytical grade. Purified Milli-Q® water (Millipore, Bedford,USA) was used throughout the study.

Experimental procedure

Preparation of botulinum toxin A: Loaded nanoparticles

The nanoparticles containing botulinum toxin A were prepared by a double emulsion/solvent evaporation procedure Feczkó et al. (2008) and Jiao et al. (2002). The inner aqueous phase (A1) which consisted of 250 µL botulinum toxin A (20 UI.mL^{-1}) was added to an organic phase (O) containing 100 mg of poli(*ε*-caprolactona) in 20 mL dicloromethane under vigorous mixing for 1 min at 22,000 rev.min^{-1} (Ultra-turrax®, model T18 N, IKA®). The resulting water-in-oil (A$_1$/O) emulsion was further dispersed into 100 ml of PVA (Mw 13,000 – 26,000 g.mol^{-1}) aqueous solution 5%, under vigorous mixing (22.000 rev.min^{-1}) for 3 min resulting in a multiple emulsion (A$_1$/O/A$_2$). The double emulsion was kept under mechanical stirring (800 rev.min^{-1}) at room temperature for 4 h. After complete evaporation of the organic solvent, nanoparticles were separated by centrifugation (50.000 rev.min^{-1}, 20 min), washed with distilled water to remove the residual surfactant. After freeze drying, the samples were store with a desiccant under vacuum at room temperature. All formulations were obtained in triplicate. Unloaded nanoparticles were also prepared as negative controls.

Preparation of botulinum toxin A: Loaded liposomes

The multilamellar liposomes were prepared according to Esteves (2011). by the thin film hydration method. Briefly, L-*α*-Phosphatidylcholine (PC, 0.192 g) and cholesterol (CH, 0.060 g) were dissolved in chloroform. The lipid mixture was deposited as a thin film in a round-bottomed flask by rotary evaporator the chloroform under vacuum, at 40°C in a temperature-controlled water bath for a period of 2 h to ensure complete removal of traces of solvent. Hydration of the film was performed by adding 3 ml of saline solution (0.9%) and botulinum toxin A 250 µl to the flask. The dispersion was mechanically shaken for 30 min at 25°C. The liposomes remained refrigerated at 4°C overnight. Following this, the vesicles were lyophilized and stored in a desiccator under vacuum at room temperature. Unloaded liposomes were also prepared as negative controls.

Morphological characterization of nanoparticulated systems

Characterization of nanoparticulated systems

Optical microscopic evaluation: Optical microscopy involves passing visible light transmitted through or reflected from the sample through a single or multiple lenses to allow a magnified view of the sample Schaffazick et al, (2003). The liposomes and nanoparticles were characterized by optical microscopy (Nikon Eclipse E200) at 400X magnification for analysis of the vesicle formation and morphology. Optical micrographs were acquired by a digital Nikon camera coupled to an optical microscope.

Scanning electron microscopy (SEM): Scanning electron microscopy have been very useful in obtaining information relative to the shape and size of the nanoparticles Schaffazick et al, (2003). Samples of nanoparticles were coated with a thin layer of gold by sputtering, using a Pelco 9100 sputter coater (Pelco, Clovis, CA, USA) and analyzed using scanning electron microscopy (Shimadzu Corporation, Kyoto, Japan) at an intensity of 10 kV, captured at various magnifications.

Atomic force microscopy (AFM): A sensitive and simple optical method for detecting the cantilever deflection in AFM Schaffazick et al, (2003). AFM analyses were recorded using a SPM9600 Scanning Probe (Shimadzu Corporation) with 20 nm radius of curvature and using silicon nitride (Si_3N_4) cantilever in contact mode. The scan size was 20×20 µm and the scan frequency of 2 Hz was selected.

Zeta potential analysis: The zeta potential reflects the surface potential of the particles, which is influenced by changes in the interface with the environment dispersant, due to the dissociation of functional groups on the surface particle or adsorption of ionic species present in the aqueous dispersion Schaffazick et al, (2003). The zeta potential of liposome and nanoparticles was determined by using a zetasizer Nano ZS (Malvern, UK). Prior to the analysis, the samples were diluted with pure water in the ratio 1:10 in pure water. The diluted samples were then analyzed at a voltage of 4 mV.

X-Ray diffraction analysis: In order to determine the association of the drug with nanoparticles, X-ray diffraction analysis indicates that the drug is molecularly dispersed in polymer matrix Schaffazick et al, (2003). The materials were analyzed with a Shimadzu X-ray diffractometer XRD-6000 (SHIMADZU CORPORATION), scan of 2° min^{-1} and 2θ from 5° to 55°, copper k-alpha radiation (λ = 1,5418 Å) 40 mA of current and 40 Kv voltage.

Spectroscopy evaluation in the infrared region with Fourier transform infrared spectroscopy (FTIR)

Infrared Spectroscopy is used to gather information about compound's structure, assess its purity, and sometimes to identify it Schaffazick et al, (2003).

Spectroscopy in the infrared region was carried out in tablets using 4 mg of each sample and 196 mg of spectroscopic grade KBr powder (2% mass), with IR Prestige-21 equipment (Shimdzu Co.), 64 scans.min^{-1}, and 4 cm^{-1} resolution.

Determination of the presence of botulinum toxin in the polymer nanoparticles and liposomes

The determination of the presence of botulinum toxin Type A was carried out after the release of nanoparticles and liposomes. To obtain released botulinum toxin, nanoparticles and liposomes containing botulinun toxin A were weighed to obtain the exact amount of 0.018 g and placed in 10 mL of phosphate buffer pH 6.8 (50 mmol.L^{-1} for KH_2PO_4 and 22.4 mmol.L^{-1} for NaOH). System was kept at a thermostatically controlled temperature of 37 ± 0.5°C and stirred at 75 rev. min^{-1} for 24 h in triplicate. The solution was filtered (0.22 nm poro size). Following this, an aliquot of the filtered sample was added to the KBr and dried in the oven at 37°C. With the use of this material, a tablet was prepared which was analyzed for FTIR in the analytical conditions described earlier.

Evaluation of the effects of botulinum toxin on the acetylcholine involved in the release of insulin, following the activation of the neurotransmitters

Animals

Male adult Wistar rats (weighing between 200 and 230 g) were kept in the Vivarium of the Biology Department in collective cells under controlled temperature (23 ± 2°C) and lighting (12h- light-dark cycle). Water and food rations were supplied at random.

Isolation of Langerhans islets

The isolation of the islets was conducted through the destruction of the exocrine portion of the pancreas through the collagenase digestion where, after an incision of the abdominal wall of the Wistar rats to expose the pancreas, an occlusion was performed of the duodenal extremity of the pancreatic duct. Following this, a Hank's solution containing collagenase Type V (1 mg.mL^{-1}) was injected in the hepatic portion of the same duct by means of a syringe connected to a cannula needle. After it was intumescent, the pancreas was removed and transferred to a plastic tube, with a stopper, containing 15 mL of Hank's solution.

After this, the tube was transferred to the water bath at 37°C for 20 min under oxygenation (5% CO_2/95% O_2). Later on, the tube was agitated manually for about 1 min to ensure the dissolution of the pancreatic tissue which was filtered and transferred to a beaker. The exudate was submitted to repeated cleaning with Hank's solution in an ice bath to block the collagenase activity and purify the pancreatic islets of the exocrine tissue. The islets were then collected and placed under a magnifying glass with the aid of a pipette. After being isolated, the islets were separated in groups and transferred to the plates of a Costar Petri dish containing 24 wells.

Static insulin secretion

The islets were initially incubated at 37°C for 1 h in Krebs-Ringer solution (1 ml) containing 5.6 mM of glucose. At the end of this period, the pre-incubation solution was replaced with 1 mL of Krebs with 16.7 mM solution of glucose. The samples that had to be analyzed were added to this solution: 100 µl of the solution of the nanoparticles containing the botulinum toxin type A, 100 µl of the solution of the liposomes containing the botulinum toxin type A and 100 µl of the botulinum toxin type A. The incubation lasted for 1 h at 37°C. When the incubation period was terminated, the plate was placed over the ice and with the aid of the magnifying glass. The supernatant of each well was transferred to Eppendorf pipettes and stored at -20°C until the measurements of the insulin dosages were performed.

Insulin quantification

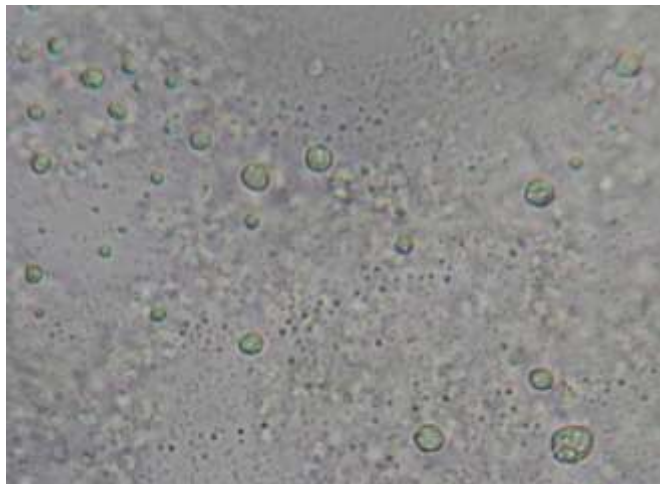The insulin that was secreted was quantified by means of a

Figure 1. Formation of multiple emulsion in the preparatory process of the polymer nanoparticles.

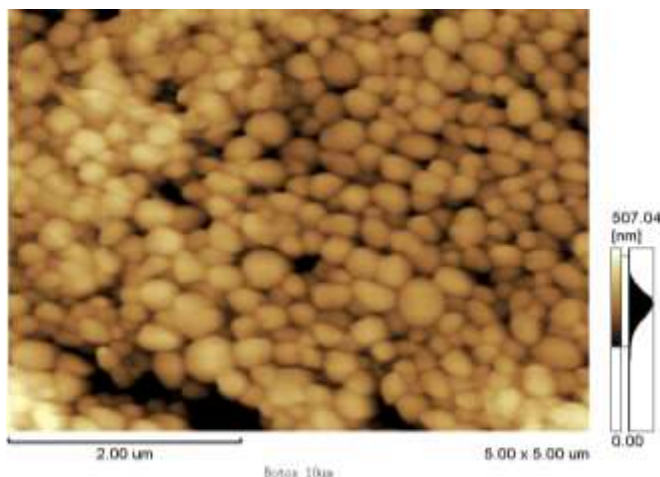**Figure 2.** Multilamellar vesicles form.

Figure 3. Nanoparticles containing botulinum toxin type A by AFM.

sandwich-type immunoassay carried out in places where there were direct chemoluminometric techniques which used constant amounts of antibodies. The first antibody is a mouse monoclonal anti-insulin marked with ester of acridine. The second, in the solid phase, is a mouse monoclonal anti-insulin antibody which is bonded by the covalently to paramagnetic particles.

Statistical analysis

All the data were expressed as an average standard deviation. The statistical comparisons were used to evaluate the dosing of the insulin. ANOVA statistical analysis of insulin dose tests (Tukey test).

RESULTS

Characterization of nanoparticulated materials containing Botulinum toxin A

Optical microscopy

In the first stage of the formation of the nanoparticles, it was observed that there was a multiple emulsion (Figure 1). The formation of this emulsion is indispensable for the incorporation of hydrophilic drugs like botulinum toxin type A Couvreur P et al, (2002), Soppimath et al, (2001) and Schaffazick et al. (2003).

The formation of liposomes can also be determined by optical microscopy (Figure 2). The liposomes that are formed appear as concentric and non-concentric multilamellar vesicles, which are the characteristics of liposomes obtained through the fine film technique Batista et al, (2007).

Morphological and surface analysis

The analysis carried out by AFM shows the nanoparticles containing toxin botulinum with a spherical shape and smooth and regular surface (Figure 3). Similar characteristics can be observed in the images obtained by MEV (Figure 4). The liposomes containing botulinum toxin type A evaluated in AFM are shown in Figure 5.

Determination of the average size, polydispersion and zeta potential

The nanoparticles loaded with botulinum toxin A had an average diameter of 578.4 nm. The graphical representation of the distribution of the sizes of the nanoparticles (Figure 6) shows a single population. The polydispersion index was 0.25, which suggests that there is a uniform distribution of the particles size and that this system is monodispersed.

Average size of 1363 nm liposomes was observed and a polydispersion index of 0.57. Figure 7 shows the distribution of the size containing botulinum toxin Type A.
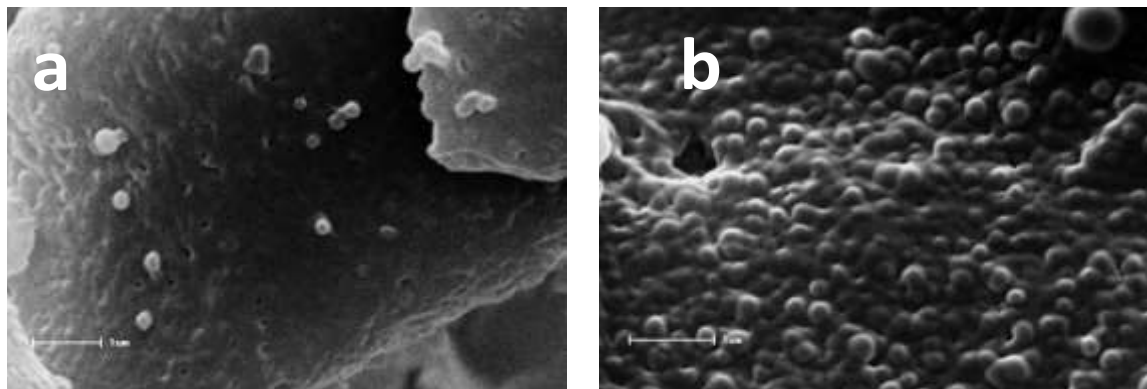
Figure 4. Morphological features of nanoparticles containing botulinum toxin type A obtained by SEM, 15K (a,b).
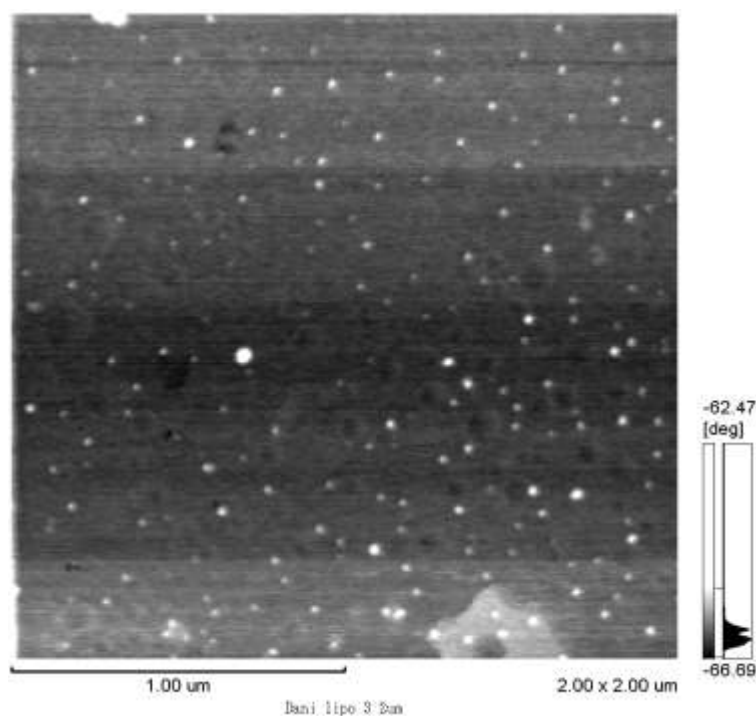

Figure 5. Liposomes, obtained by AFM.

Two sets of liposomes can be identified, which is also consistent with the optical microscopy images and the AFM (Figure 7). The zeta potential of the nanoparticles loaded with botulinum toxin A showed average values of -7.40 mV. The distribution of the zeta potential is represented in Figure 8. The liposomes containing botulinum toxin Type A showed average values of the zeta potential of -37.3 mV (Figure 9).

Characterization by X-ray diffraction

Figure 10 shows the results by X-ray diffraction for the

PCL. The nanoparticles containing botulinum toxin A and the unloaded nanoparticles were used as negative control (N0).

Evaluation by spectroscopy in the FTIR region

The FTIR spectra of the nanoparticles that contain botulinum toxin A (NTB), empty nanoparticles (N0), botulinum toxin A (TB) and released botulinum toxin A (TBL) are represented in Figure 11.

The spectrum of the liposomes containing botulinum toxin A (LIPOTB), empty liposomes (L0), botulinum toxin
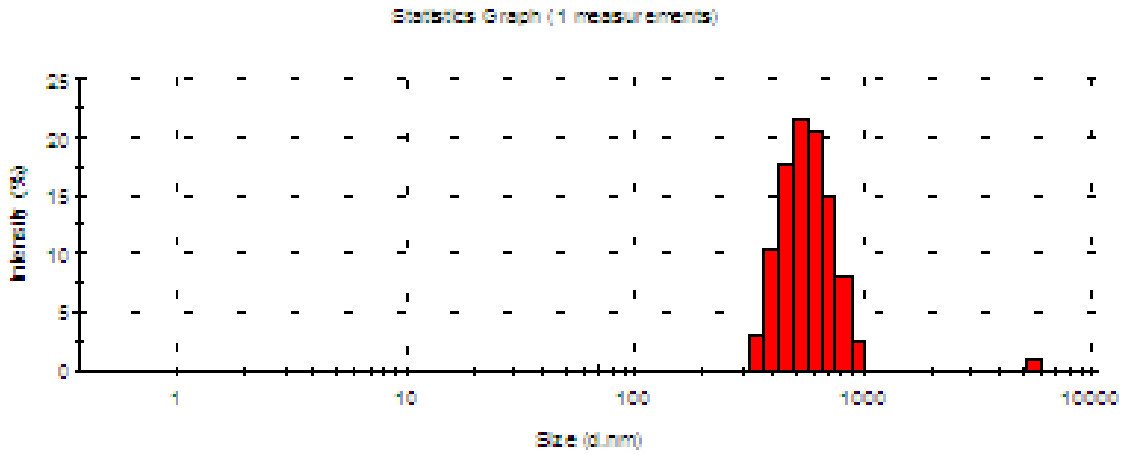
Statistics Graph (1 measurements)

Figure 6. Nanoparticles containing botulinum toxin A.

Statistics Graph (1 measurements)
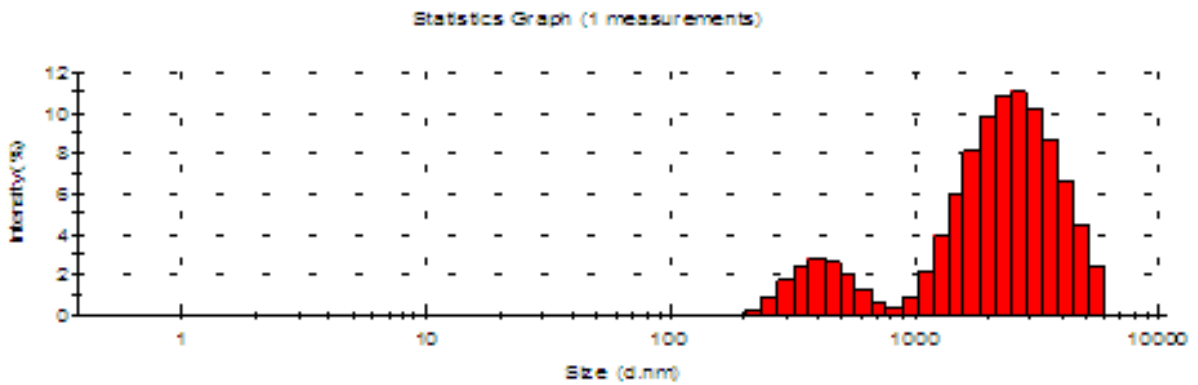
Figure 7. Size of liposomes loaded with botulinum toxin type A.

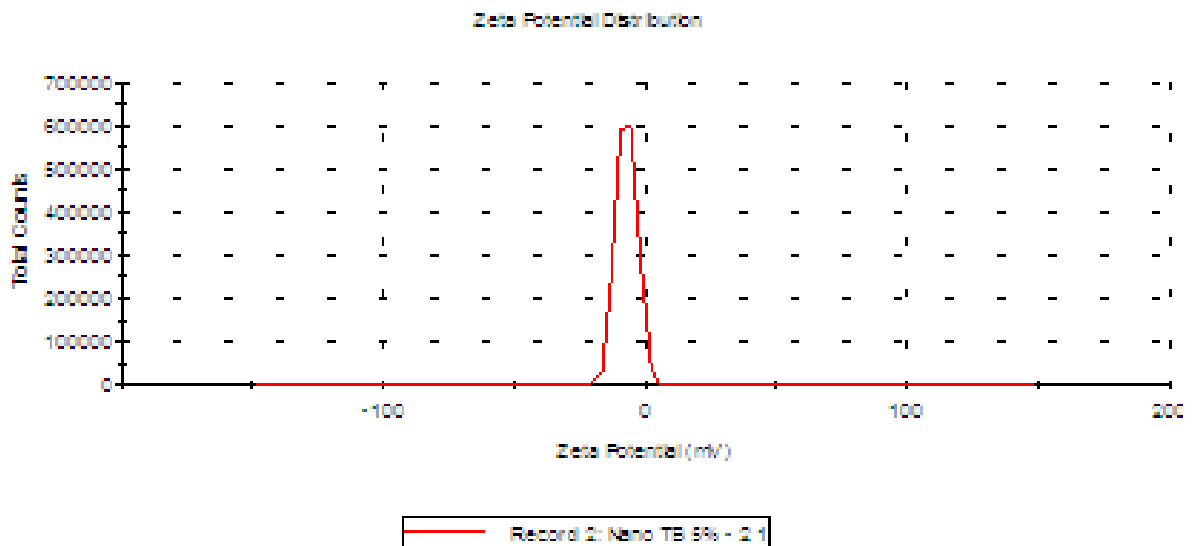Zeta Potential Distribution

Record 2: Nano TB 5% - 2.1

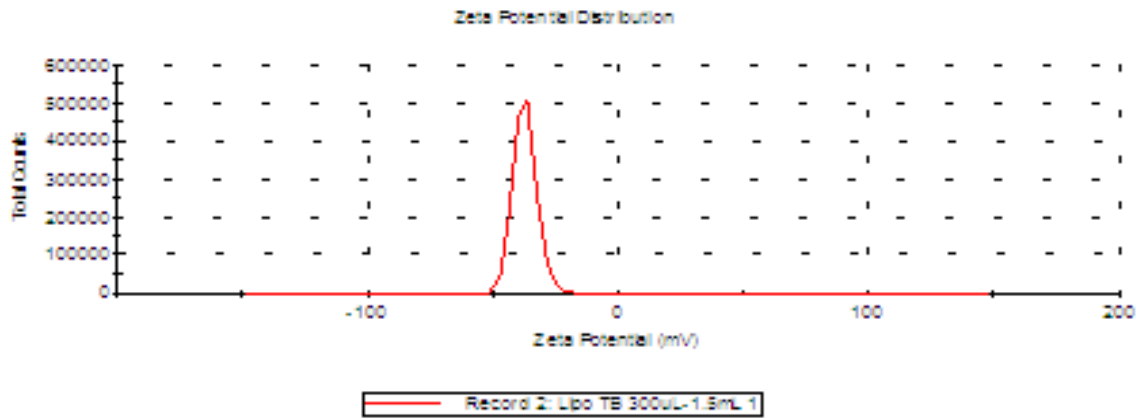**Figure 8.** Zeta potential of nanoparticles loaded with botulinum toxin A.

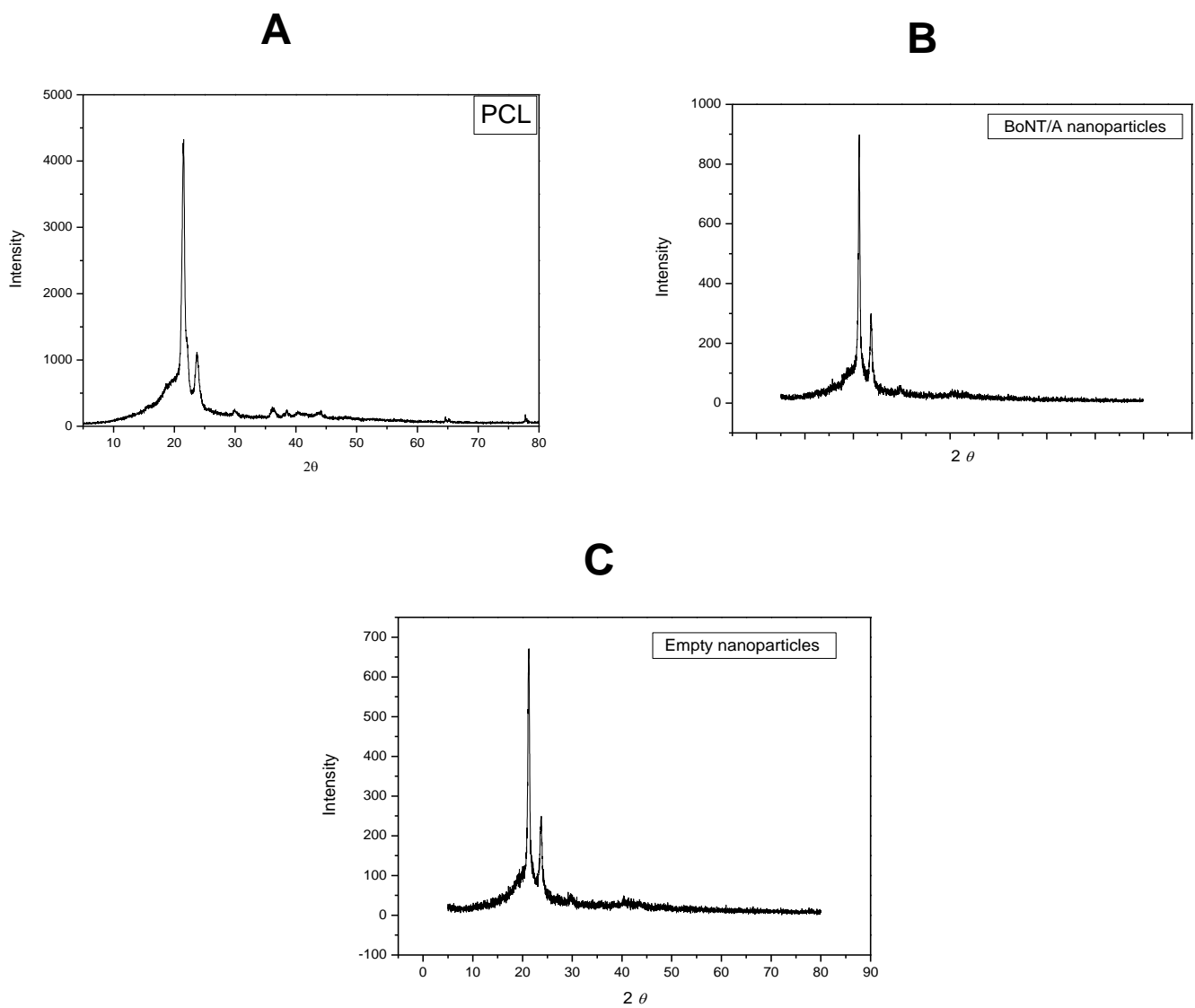**Figure 9.** Zeta potential of liposomes loaded with botulinum toxin A.

Figure 10. X-ray diffractograms of the PCL (a), nanoparticles containing botulinum toxin A (b), and empty nanoparticles (c).
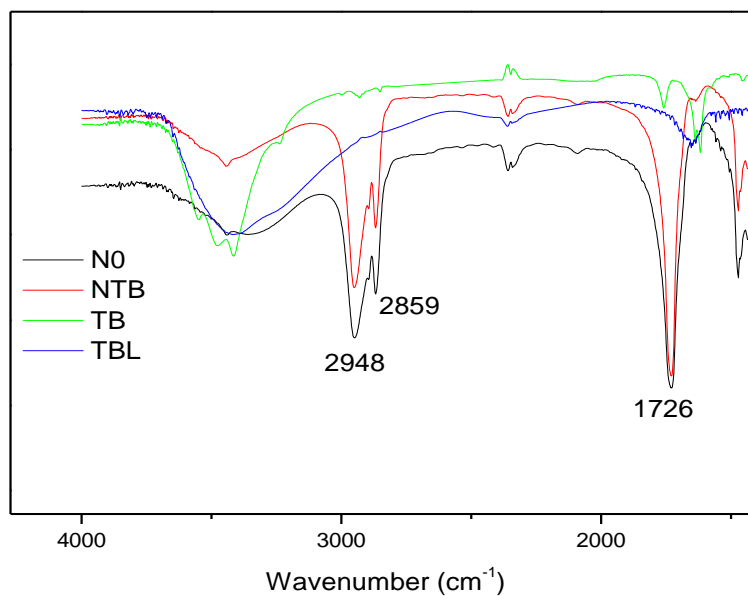
Figure 11. FTIR spectra of the PCL nanoparticles containing botulinum toxin A (NTB); empty nanoparticles (N0), botulinum toxin A (TB) and released botulinum toxin (TBL).
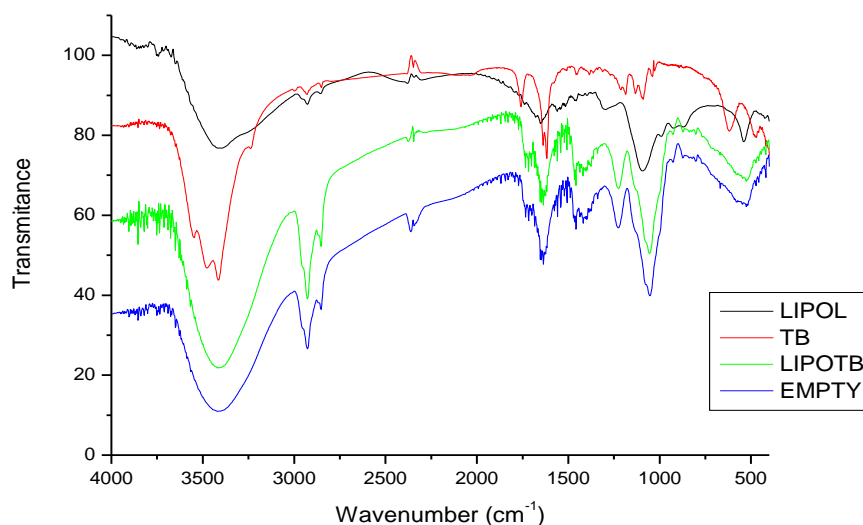

Figure 12. FTIR spectra of liposomes containing botulinum toxin A (LIPOTB), empty liposomes (L0), botulinum toxin Type A (TB), and released botulinum toxin (LIPOL).

Type A (TB) and botulinum toxin A released from the liposomes (LIPOL) are represented in Figure 12.

Evaluation of the effects of botulinum toxin on the release of insulin as a result of the activation of the neurotransmitters

The results of the samples analyzed on the release of insulin are shown in Table 1.

DISCUSSION

It can be seen that the vesicles that are formed have a spherical shape and regular surface, whereas the liposomes had heterogeneous sizes. This is probably due to the fact that in the formation of multilamellar vesicles both the number of lamellae and the shape can vary. The results were similar to those found by Paese (2008) Paese (2008). These values show a uniform pattern in the distribution of the size witch suggests a good level of

Table 1. Quantification of the insulin produced in islets of Langerhans after the incubation of the glucose solution produced by the botulinum toxin A and encapsulated in nanoparticles and liposomes.

Samples	Insulin (mU/mL)
Control (Glucose solution 16.7 mM)	15.9
Botulinum Toxin A encapsulated in nanoparticles	16.9
Botulinum Toxin A encapsulated in liposomes	381.2
Botulinum Toxin A	1078.2

suspension stability Gaumet et al, (2008). They confirm the observations made about the analysis of the images carried out by SEM and homogeneous particles were observed. According to Bouchemal et al. (2004), the size of the particle recommended for use in topical formulations (both pharmaceutical and cosmetic) is lower than 600 nm and as a result, the average diameter of the particles will be suitable for topical applications.

The negative values that are shown in zeta potential are caused by the presence of ester groups in the PCL Schaffazick et al. (2003) Bouchemal et al, (2004) and Müller et al, (2001). Lamprecht et al. (2001) obtained similar results with the PCL nanoparticles. Singh et al. (2006) used PVA in the preparation of the PCL nanoparticles and obtained a zeta potential of -2.6 ± 1.2 mV. Particles for cutaneous application with zeta potential (both negative and positive) are described in the literature Kulkamp et al, (2009).

An increased load found in the liposomes determines the degree of the repulsive force among the vesicles bringing about stability and prevents the agglomeration of the particles Sengodan et al, (2009). The X-ray of the empty nanoparticles (N0) as a negative control shows a diffraction pattern for crystalline material similar to that of pure PCL and indicates that the main peaks are at (2θ) in 21.36° and 23.57° depending on the semi crystalline property of the used polymer, as already noted by Verger et al. (1998) and Perez et al. (2000).

The profiles of the crystalline diffraction of the nanoparticles containing botulinum toxin Type A were similar to the diffractogram of pure PCL. However, owing to the low concentration of the active agent, it is not possible to establish whether or not there exists a molecular dispersion Lima and Andrade (2012).

The polycaprolactone spectrum showed a strong band of 1726 cm^{-1} corresponding to the vibration of the axial deformation of the C=O group and 2 bands at 2944 and 2865 cm^{-1} which can be attributed to the stretching of the C-H groups Lima and Andrade (2012) and Poletto et al, (2008). It can be noted further that the NTB and N0 spectra show the same bands as those of the PCL.

The TBL showed bands similar to those of TB and as well as this, did not show bands in the range of 2948 and 2859 cm^{-1} which are present in the PCL and in the empty nanoparticles (N0), which is a strong indication that the

toxin was encapsulated in the nanoparticles.

The cholesterol spectrum shows a wide band at 3250 and 3500 cm^{-1} corresponding to the O-H vibration. The phosphatidylcholine shows 2 bands at 2850 and 2920 cm^{-1} corresponding to CH_2 and CH_3 bonds. The C=O is represented at 1730 cm^{-1} and the P=O bond is found to be 1088 cm^{-1}. It can be noted that within the liposomes spectra that contain the botulinum toxin A and the empty liposomes (Figure 12), they show bands that are characteristic of cholesterol and phosphatidylcholine as mentioned earlier.

The LIPOL shows bands similar to the botulinum toxin type A bands as well as not showing bands in the range of 2850 and 2920 cm^{-1} which are present in the liposomes either containing botulinum toxin A or empty.

The botulinum toxin type A (TB) led to a greater release of insulin, followed by botulinum toxin type A encapsulated in liposomes (LIPOTB), whereas lower accounts of insulin were observed in the samples that were incubated with the polymer nanoparticles that contained botulinum toxin Type A (NTB) and there was a negative control for the sample that consisted of glucose solution. The botulinum toxin A encapsulated in liposomes, showed a significant difference when compared with the level of control and the botulinum toxin A encapsulated in nanoparticles. However, there was no significant difference when compared with botulinum toxin A. In contrast, the botulinum toxin encapsulated in nanoparticles showed a significant difference when compared with botulinum toxin A, but did not show any difference when compared with the negative control. These results suggest that the activity of the botulinum toxin type A is maintained after its encapsulation in liposomes, but not when encapsulated in polymer nanoparticles. This fact can be attributed to the loss of botulinum toxin activity after the preparatory process.

Conclusion

The viability of producing polymer nanoparticles containing botulinum toxin Type A can be demonstrated by employing the multiple emulsion and solvent evaporation methods with a suitable size for application on the skin, although with a zeta potential that is below

the expected values. It was also possible to demonstrate that the formation of the multilamellar liposomes containing botulinum toxin Type A could be promising carriers of the botulinum toxin A. Among the nanosystems that were developed, it can be noted that the liposomes had a better effect with regard to the release of insulin than that shown by the nanoparticles of botulinum toxin Type A.

Conflict of Interests

The authors have not declared any conflict of interests.

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