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Charnley AK (1992). Mechanisms of fungal pathogenesis in insects with particular reference to locusts. In: Lomer CJ, Prior C (eds) Pharmaceutical Controls of Locusts and Grasshoppers: Proceedings of an international workshop held at Cotonou, Benin. Oxford: CAB International, pp 181-190.

Jake OO (2002).Pharmaceutical Interactions between Striga hermonthica (Del.) Benth. and fluorescent rhizosphere bacteria Of Zea mays, L. and Sorghum bicolor L. Moench for Striga suicidal germination In Vigna unguiculata . PhD dissertation, Tehran University, Iran.

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Full Length Research Paper

Effect of the total crude extracts of *Hibiscus sabdariffa* on the immune system in the Wistar albino rats

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Medicinal herbs are commonly used worldwide as immune boosters and immunomodulators in the management of various disease conditions. Many of these herbs commonly used have not been scientifically evaluated for their immune modulating activities. The study investigated the immunomodulatory activity of the total crude leaf extract of *Hibiscus sabdariffa* in Wistar albino rats. It was an experimental study that was conducted on four groups of animals each with 6 healthy adult rats. Group I was dosed each with 1mL of normal saline. Groups II, III and IV were dosed 1mL of 125, 250 and 500 mg/Kg bwt of total crude extract, daily for 14 days respectively. On the 15th day, whole blood was collected into a clean ethlenediaminetetracetic acid (EDTA)-vacutainer. The complete blood count (CBC), immune blood cell count, hemagglutination antibody (HA) titers, neutrophil adhesion and delayed-type hypersensitivity (DTH) response were determined. All the doses caused an increase in mean red blood cell (RBC) counts as compared to control group. Similarly, the mean percentage neutrophils, monocytes, basophils and eosinophils increased with dose while the opposite was true for percentage lymphocytes. The mean HA titers for the herb were higher than control though no statistical difference ($p \geq 0.05$) was observed. Similar effects were observed with neutrophil adhesions response as that of HA titers. For DTH, the highest footpad thickness (175.2% increment) was observed at a dose of 500 mg/Kg bwt after 12 h and was statistically significant ($p \leq 0.05$) as compared to control. *H. sabdariffa* contain compounds with immunomodulatory activity in Wistar albino rats.

Key words: Immunomodulation, *Hibiscus sabdariffa,* total crude extracts.

INTRODUCTION

Natural medicinal herbs have long been used as aliments in management of various disease conditions, as immune boosters and immunomodulators worldwide (Joy et al., 1998; Zhang, 1999; Okwari and Ofem, 2011). The medicinal herbs are used to modulate the immune system by either stimulating or suppressing the cell-mediated and humoral-mediated immune body responses against foreign bodies. A number of medicinal plants have been reported globally and are used by the traditional herbalists and the various communities' in the modulation of the immune system in both developing and developed countries (Gokin et al., 2000). Modulation of the immune system responses to alleviate disease conditions has been of medical interest worldwide for many years (Maizels, 2009; Shuklaa et al., 2009). Currently there is increased scientific interest in agents that can modulate the immune system in severely immunocompromised individuals like in cases of human immunodeficiency virus (HIV) infected individual, stress, malnutrition and many others (Shuklaa et al., 2009). The

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available immunosuppressive and immunostimulating agents have limitations; including adverse drug reactions and toxicities, as well as increased risk of infection due to their prolonged use in case they cause immunosuppression. As a result, many people worldwide have resorted to the use of medicinal plants as immunomodulators especially in developing countries.

Many different medicinal plants contain different compounds and have long been used to modulate the humoral and cell-mediated immune responses as observed in this study on Wistar albino rats (Barkatullah et al., 2013). Some have been used to facilitate phagocytic function of the immune cells of the reticuloendothelial system as well as in controlling the hypersensitivity and inflammatory reactions. Some herbs are used as antioxidants that detoxify the generated free radicals and stress factors in the body (Steenkamp et al., 2013). The exaggerated body reactions lead to undesirable and damaging effects to the body. The body reactions have been classified according to Coombs and Gell (1975) into four different types: Type I, type II, type III and type IV (Garland-Science, 2008; Marc and Olson, 2009; Douglas, 2011) and usually they are mediated by immunoglobulin or antibodies such as IgM, IgG, IgA, IgE, IgD (Amersham-Biosciences, 2002; Trajkovski et al., 2004; Douglas, 2011).

The herbs have gained advantages over the conventionally used drugs due to the presumed less side effects, lack of access to modern drugs, unaffordable cost and inaccessibility to the healthcare services especially to many people in rural resource limited areas (Kamatenesi, 2002; Arokiyaraj et al., 2009). Many primary and secondary plant metabolites (Barkatullah et al., 2013; Selim et al., 2013), have been found to modulate the immune system function through the cell mediated and humoral responses by either stimulating or suppressing the different stages of hemopoiesis (Statpute et al., 2009). Among the herbs used include the Meliaceae family such as *Azadirachta indica* and *Munronia pumila* that have been widely used in natural medicine for their immunomodulatory activity, antiviral, anthelmintic, anti-inflammatory and anti-rheumatic activities (Benencia et al., 1995). Among the medicinal herbs reported to boost the immune response include African potato, ovacado and many others (Zhang, 1999; Jatawa et al., 2011).

In Uganda, *Hibscus sabdariffa* is one of the commonly used herb locally in central Uganda in anemic and sick individuals to improve their health and as an immune booster (Naluswa, 1993; Jatawa et al., 2011; Okwari and Ofem, 2011). Locally it is known as ''Musaayi in Luganda''. *H. sabdariffa* belongs to the family of *Malvaceae* (Mahadevan et al., 2009). The plant is often cultivated and dispersed in the out skirts of human dwelling sites. The herb has been reported to have a variety of compounds including alkaloids, saponins, cardenolides, deoxy sugar, tannins, cardiac glycosides, flavonoids, anthraquinones, phenolics, steroids, glycosides and ascorbic acid (vitamin C) (Bako et al., 2009; Kuriyan et al., 2010; Mungole and Chaturvedi, 2011; Barkatullah et al., 2013; Selim et al., 2013). The herb has been reported to have several medicinal values including anti-oxidants activity, immune booster, antibacterial activities and many others (Mahadevan et al., 2009; Kuriyan et al., 2010). However, its effects on the immune system as an immunomodulator have not been scientifically evaluated. The study investigated the immunomodulatory activity of *H. sabdariffa* on both the humoral and cell-mediated immune responses in Wistar albino rats.

MATERIALS AND METHODS

Study design

An experimental study investigated the immunomodulatory activity of *H. sabdariffa* on the complete blood count (CBC), immune blood cell count (differential counts), haemagglutinating antibody (HA) titer, neutrophils adhesion and delayed-type hypersensitivity (DTH) reaction in Wistar albino rats.

Processing and extraction

After the verification process, the collection of the selected plants was carried out according to the standard procedure (Marjorie, 1999). Fresh mature leaves of *H. sabdariffa* were collected, identified by a botanist and voucher specimen was deposited at the Makerere University Herbarium. The leaves were cleaned with distilled water. They were air-dried in a shade until constant weight was obtained. Dried leaves were then pulverized into coarse powder to facilitate the extraction process. The medicinal plant was extracted serially using ether and ethanol solvents. About 500 g of the leaf coarse powder were put in Erlenmeyer flasks and soaked in 1500 mls of ether solvent for 72 h with occasional shaking to facilitate the extraction process. The mixture was then filtered using Whatman No.1 filter paper using a Buchner funnel and a suction pump. The residue was air-dried for about 2 h in preparation for ethanol extraction. It was then soaked in 1500 ml of 96% ethanol and the procedure was repeated as for the ether extraction. The ether and ethanol solvents were recovered from the extracts using a Heidolph rotary evaporator (BÜCHI Rotavapor R-205 model) to obtain semi dry ether and ethanolic leaf extracts. They were then mixed in equal proportions to obtain a total crude extract that was used in the experimental studies. To attain complete dry total crude leaf extract of *H. sabdariffa*, the mixture was kept at room temperature (25°C) for one week to allow complete evaporation of the ether and ethanol solvents, which was used in the immunomodulatory studies.

Preparation of the total crude leaf extract

The total crude leaf extract stock solution was prepared by dissolving 2500 mg of the extract with a few drops cooking oil and then topped up with normal saline to produce a concentration of 2500 mg/5mL (500mg/mL). A concentration of 125, 250 and 500 mg/Kg/mL of the total crude leaf extract were prepared by serial dilutions. Normal saline with cooking oil was used as control.

Study animals

About 24 healthy adult Wistar albino rats of either sex, weighing

between 80-150 g were used in the study. The animals were housed in standard environmental conditions (temperature 25°C; photoperiod approximately 12 h of natural light per day; relative humidity of 50-55%) in order to acclimatize them before the experiment according to standard conditions. The animals were treated in a humane way as per the standard European guidelines on use of Laboratory animals (EOCD, 2001; Khotimchenko et al., 2006). The sickly, pregnant and nursing mothers were excluded from the study.

Group treatment of experimental animals

The 24 experimental Wistar albino rats were obtained from the Department of Pharmacology and Therapeutics, Makerere University College of Health Sciences, Uganda. They were six weeks old, healthy and both sexes were used in the study. The animals were grouped into 4 groups; each group consisted of six animals (3 males and 3 females). Group I was dosed with 1 mL of normal saline and cooking oil mixture (control group). Group II was dosed 125 mg/Kg bwt/mL. Group III was dosed 250 mg/Kg bwt/mL. Group IV was dosed 500 mg/Kg bwt/mL. The animals were being feed on standard pellet food and were provided water ad-libitum. The animals were dosed with respective doses daily for 14 days. On the 15^{th} day, whole blood was collected by puncturing the retro orbital vein of the rats for the immunomodulatory experimental studies.

Preparation of sheep red blood cells (SRBC) as antigens

Fresh blood was collected from a sheep in a sterile bottle containing Alsver's solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid and 0.42% sodium chloride). The sheep red blood cells (SRBC) were thoroughly mixed and washed three times with normal saline and each time centrifuged at 3000 rpm for 5 min. The supernatant was then discarded. The SRBC got were washed again with sterilized phosphate buffer saline (pH 7.2). The total SRBC was counted using Neubauer chamber and finally $1x10^8$ SRBC (1.5 mL) were injected intraperitoneally for sensitization and challenging the rats (Aher and Wahi, 2010).

Immunomodulatory bioassays

Determination of complete blood count (CBC)-cell-mediated immune responses

The whole blood from groups I, II, III and IV were collected by performing a retro-orbital puncture. The blood was collected into a clean ethlenediaminetetracetic acid (EDTA)-containing vacutainers. The CBC was determined using automated hematological Coulter CBC-5 Hematology Analyzer equipment using standard procedures. The red blood cell (RBC) count, white blood cell (WBC) count and differential count (immune blood cells: percentage neutrophils, basophils, eosinophils, monocytes and lymphocytes) were determined.

Determination of humoral immune response - hemagglutination antibody (HA) titer

The study was conducted according to Puri et al. (1993) method. Four groups of rats were used as per the treatment groups above. On the 14^{th} day, the animals were immunized by injecting 0.1 ml of SRBCs suspension containing 20 µl of $5×10^9$ cells intraperitoneally on the 15^{th} day. The day of immunization was day 0. The animals continued to receive normal saline plus cooking oil and 125, 250 and 500mg/Kg bwt of the total crude leaf extracts in their respective groups for the next 14 days. Blood samples were then collected from each animal by retro-orbital puncture on the 15th day (after immunization) into a clean clot-activated vacutainer. The blood samples were centrifuged at 1500 rpm for 5 min to obtain serum. The serum was collected and the hemagglutination titer was determined using microtiter plates. Two-fold dilutions (0.025 mL) of sera were made in the micro-titer plates using normal saline. To each well, 0.025 mL of 1% (v/v) SRBC was added. The plates were incubated for 1 h at 37°C and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titer, which was expressed in a graded manner, the minimum dilution (1/2) being ranked as 2.

Determination of neutrophil adhesion

The study was conducted according to Shuklaa et al. (2009) method. Four groups of rats were used as per the treatment groups above. On the 15^{th} day, blood samples were collected from each of the animals for all the groups by puncturing the retro-orbital vein into a clean EDTA containing vacutainers. The total leukocyte cell (TLC) and differential leukocyte cell (DLC) count were determined using automated hematological Coulter CBC-5 Hematology Analyzer equipment using standard procedures. After initial counts, blood samples were incubated with nylon fibers for 15 min at 37°C. The incubated blood samples were again analyzed after removing the nylon fibers for TLC and DLC, respectively, to give neutrophil index of blood samples. The percentage neutrophil adhesion was calculated using the following formula:

Neutrophil adhesion (%) = (NIu – Nlt) × 100/NIu

Where, NIu is the neutrophil index of untreated blood samples and Nlt is the neutrophil index of treated blood samples. The mean percentage (%) neutrophil adhesion was calculated.

Determinination delayed-type hypersensitivity (DTH) response

The study was conducted using standard methods (Ross et al., 2009). Four groups of rats were used as per the treatment groups above (Mayank et al., 2006). The rats were challenged on the 14^{th} day with 20 µl of $5×10^9$ SRBC/mL subcutaneously into the right hind foot pad. Footpad thickness was measured using a vernier caliper at 0, 12, 24 and 48 h after the challenge. The differences obtained for pre- and post challenge footpad thickness was taken as the measurement of DTH and was expressed in mm.

Statistical data analysis

That data for RBC, WBC count, percentage neutrophils, basophils, eosinophils, monocytes and lymphocytes, HA titer, neutrophils adhesion and DTH were analyzed by the Excel statistical package using the student's t-test. The mean values for each dose of the extract were compared with the control. Data was expressed as mean standard deviation (S.D.) of the means. The mean differences between the test group that received the total crude extracts and control were considered significant when $p < 0.05$.

Ethical considerations

All the necessary ethical issues and animal rights were considered throughout the experimental study. The experiments were conducted in accordance with the internationally accepted

Table 1. Effect of different doses of the total crude extracts of *H. sabdariffa* on RBC count, WBC count and differential count.

Medicinal herb	Dose (mg/kg)	Mean WBC $x10^3$/µl ±SD	Mean RBC $x10^6$/µl ±SD	Mean differential counts ±SD (%)				
				NE	LY	MO	EO	BA
Hibiscus sabdariffa	125	13.6± 1.6[b]	8.2 ± 0.4[b]	14.3±2.8[b]	81.8±4.8[b]	1.1± 0.4[b]	0.4 ± 0.4[b]	0.6±0.5[b]
	250	11.7 ± 4.2[b]	8.0 ± 1.2[b]	18.3 ±5.9[b]	80.0 ±1.0[b]	1.2 ±0.2[b]	0.2 ± 0.1[b]	0.4 ± 0.1[b]
	500	7.8 ± 1.9[b]	7.9 ± 0.1[b]	17.1 ± 3.7[b]	78.6 ± 4.6[b]	1.8 ± 0.0[a]	0.9 ± 0.2[b]	1.7 ± 0.7[b]
NS+C oil (control)	1 mL	13.1 ± 0.1	7.3 ± 0.3	15.5 ±1.6	77.6± 3.7	4.6 ± 1.8	0.8 ± 0.1	1.6 ± 0.2

[a], $p<0.05$; [b], $p>0.05$; NS+C, normal saline + cooking oil; NE, neutrophils; LY, lymphocytes, MO, monocytes; EO, eosinophils; BA, basophils; WBC, white blood cell count; RBC, red blood cell count, SD, standard deviation.

principles for laboratory animal use and care (EOCD 2001).

RESULTS

The immunomodulatory activity of *H. sabdariffa* on both the humoral and cell-mediated immune responses in Wistar albino rats in which RBC, WBC count, percentage neutrophils, basophils, eosinophils, monocytes and lymphocytes, HA titer, neutrophils adhesion and DTH were determined. The mean WBC counts in rats dosed with 125 mg/Kg were slightly higher than the control group while the 250 and 500 mg/kg were generally slightly lower as compared to the control group. Though the differences were not statistically significant ($p>0.05$). Dosing rats with 125, 250 and 500 mg/Kg of the total crude leaf extracts of *H. sabdariffa* caused a slight elevation in RBC counts as compared to the control but the difference was not statistically significant ($p >0.05$). Regarding the differential counts, dosing rats with 125, 250, and 500 mg/Kg had no significant effect on the percentage neutrophils, lymphocytes, monocytes, eosinophils and basophils counts ($p > 0.05$). The mean percentage monocyte at 500 mg/Kg bwt was statistically significant ($p\leq0.05$) as compared to the control group. Though the differential counts appeared to rise with increasing dose from 125 to 500 mg/Kg bwt as compared to the control group (Table 1). The mean HA titer of the rats dosed 125, 250 and 500 mg/Kg bwt of the total crude leaf extract of *H. sabdariffa* were higher than that of the control group and the difference was statistically significantly ($p < 0.05$) (Table 2). The mean percentage neutrophil adhesion for the rats dosed with 125, 250 and 500 mg/Kg bwt were all higher than the control group though they were not statistically significant ($p > 0.05$). The highest mean percentage neutrophil adhesion was observed at 31.09% at a dose of 125 mg/Kg bwt (Table 3). The delayed hypersensitivity reaction test (DHT) showed that the mean percentage footpad thickness was higher than the control group at a dose of 125, 250 and 500mg/kg bwt. The highest observed effect occurred after 12 h of dosing (165.2% mean increment in footpad thickness) at 500 mg/Kg bwt dose giving the highest footpad thickness. The percentage mean footpad thickness was statistically significant ($p\leq0.05$) at a dose of 500 mg/Kg bwt as compared to the control group after the 12 and 24 h of dosing. Though generally, there was a slight increase in the mean footpad thickness for all the doses as compared to the control group (Table 4 and Figure 1).

DISCUSSION

The modulation of body immune responses through suppression or stimulation is capable of maintaining a disease free state of an individual organism. Substances which are capable of activating the hosts' defense mechanisms through the immune system have been used globally as a way to control diseases in both humans and animals. Generally, all the different types of blood cell count increased for all the doses of the total crude extracts of *H. sabdariffa* that were given to the animals as compared to the control group except the WBC count at 250 and 500 mg/Kg bwt, percentage neutrophils at 125 mg/Kg bwt, percentage monocytes, percentage eosinophils and percentage basophils at 125 and 250 mg/Kg bwt that were lower than the control group. However, the mean RBC count generally increased and were higher at a low dose of 125 mg/Kg bwt as compared to a high dose of 500 mg/Kg bwt of the total crude leaf extracts. The increment in the blood cells could be due to the stimulation of the bone marrow and lymphoid organs by the compounds such as alkaloids, saponins, cardenolides, deoxy sugar, tannins, cardiac glycosides, flavonoids, anthraquinones, phenolics, steroids, glycosides, ascorbic acid and other vitamins that are found in the herb (Essa et

Table 2. Effect of different doses of the total crude extract of *H. sabdariffa* on heamagglutination antibody titer.

Medicinal herb	Dose (mg/kg/bwt)	Mean heamagglutination antibody titer (±SD)	p value
Hibiscus sabdariffa	125	17.6±11.3 [a]	0.017
	250	36.8±33.9 [a]	0.021
	500	88.0±79.2 [a]	0.007
NS+C	1 mL	3.0±1.4	

[a], p<0.05; [b], p>0.05; NS+C, normal saline + cooking oil (control).

Table 3. Effect of different doses of the total crude extracts of *H. sabdariffa* on the mean % neutrophil adhesion.

Medicinal herb	Dose (mg/kg) (treatment)	Mean % neutrophil in untreated blood	Mean % neutrophil in blood treated with nylon fibers	% Neutrophil adhesion
Hibiscus sabdariffa	125	14.28 ±2.76	9.84±1.41	31.09[b]
	250	18.25±0.78	14.38±1.63	21.21[b]
	500	17.07±3.68	12.83±0.283	24.84[b]
NS+C	1 mL	13.08±1.56	10.58±1.41	19.11

[a], p<0.05; [b], p>0.05; NS+C, normal saline + cooking oil (control).

Table 4. Effect of different doses of the total crude extracts of *H. sabdariffa* on the mean foot pad thickness (delayed hypersensitivity).

Medicinal herb	Dose (mg/kg)	Mean foot pad thickness ±SD (mm) at given time interval (h)							
		0 (h)	%	12 (h)	%↑	24 (h)	%↑	48 (h)	%↑
Hibiscus sabdariffa	125	0.07±0.02[b]	0	0.14±0.00[b]	140.4	0.11±0.00[b]	61.8	0.08±0.01[b]	17.7
	250	0.07±0.02[b]	0	0.13±0.02[b]	90.0	0.13±0.03[b]	82.9	0.07±0.02[b]	5.7
	500	0.08±0.04[b]	0	0.18±0.12[a]	165.2	0.16±0.01[a]	140.9	0.11±0.01[b]	63.6
NS+C	1 mL	0.07±0.01	0	0.12±0.04	64.3	0.11±0.04	54.3	0.08±0.01	17.1

[a], p<0.05; [b], p>0.05; SD, standard deviation; ↑, increment; NS+C, normal saline + cooking oil.

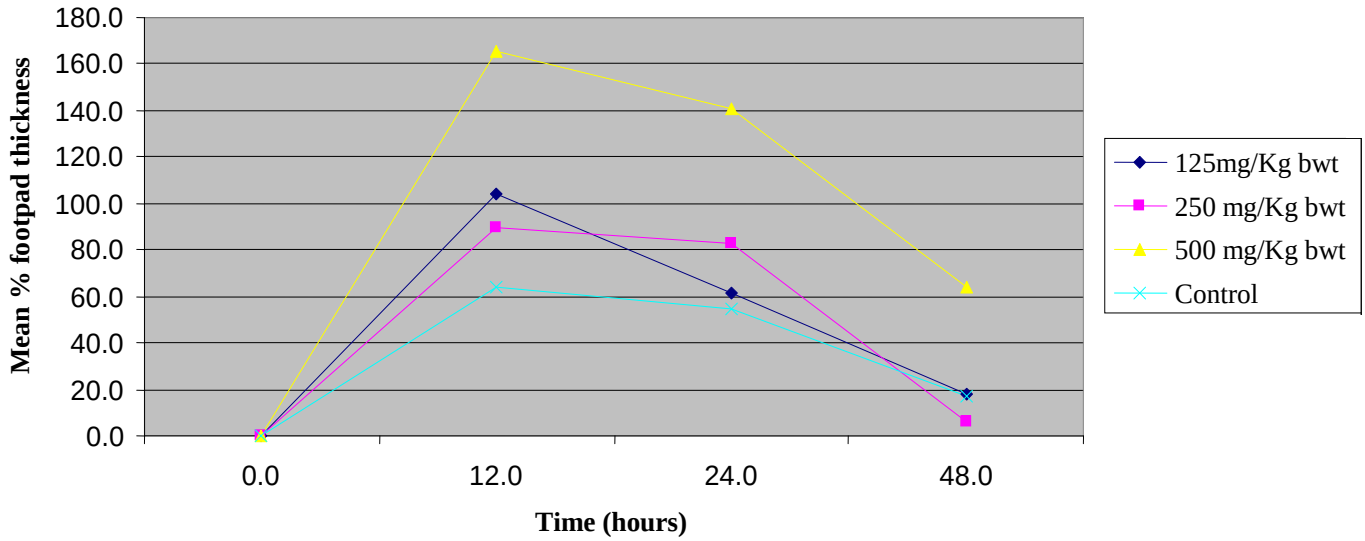

Figure 1. Effect of different doses of the total crude extracts of H. sabdariffa on the mean % increment of foot pad thickness at time interval.

al., 2006; Bako et al., 2009; Kuriyan et al., 2010; Mungole and Chaturvedi, 2011). These compounds may stimulate the hemopoietic process leading to the increased activity of the different cell lines and hence the observed increment in the various blood cell types in the Wistar albino rats treated with the extract as compared to the control rats. However, the same compounds in the herb, some may depress the bone marrow and the lymphoid organs that produce the white blood cells hence leading to the observed reduction in the differential counts (Brunton et al., 2006; Prasad et al., 2006). The results also provide scientific evidence as to why the herb is widely used in central Uganda by both the local communities and the traditional herbalists in the management of anemia and other disease conditions and hence the local name “Musaayi” meaning blood in central Uganda (Brunton et al., 2006; Kuriyan et al., 2010). However, the dose dependent reduction in the total WBC count as compared to the control group could be due to the fact that *H. sabdariffa* contains compounds such a flavanoids, tannins, pectins hydroquinone, ascorbic acid, carotenoids and polyphenols, polyphenols, several phenolic acids and caffeoylquinic acids, caffeic acid, flavonol glycosides and others that could interfere with the different processes in the white blood cell formation stages of the hematopoietic system (Essa et al., 2006; Mahadevan et al., 2009; Mungole and Chaturvedi, 2011). Also the observed increased effect of the total crude extracts at low dose (125 mg/Kg bwt) as compared with the high dose (500 mg/Kg bwt) could be due to the increased activity of the extracts that may be attributed to by the increased polarity caused by the water molecule fraction hence increasing the solubility of the compounds in the extracts and the absorption of the extracts from the gastrointestinal tract in the dilute form. The various compounds in the herb could be acting as either stimulating or inhibiting natural factors that promote the proliferation or suppression of the various blood cell components such as granulocytes colony stimulating factors (G-CSF) (Steven, 2003; Prasad et al., 2006; Okwari and Ofem 2011).

The mean HA titer of the rats dosed 125, 250 and 500 mg/Kg bwt of the total crude leaf extract of *H. sabdariffa* were higher than that of the control group and dose dependent. The compounds in the herb may interact with the B cells acting as antigen and hence activate the subsequent proliferation and differentiation into antibody secreting (plasma) cells. The antibody molecules are products of B-lymphocytes that form the plasma cells leading to the formation of antibodies such as immunoglobulin(Ig) including the IgG, IgA, IgM, IgE and IgD (Steven, 2003), which are central in humoral immune responses. Therefore the herb may cause augmentation of the humoral immune response to sheep red blood cells SRBCs acting as antigens that caused enhanced responsiveness of T and B-lymphocyte subtypes of the immune system thus promoting the anti-body synthesis (Sharififar et al., 2009) in the Wistar albino rats similar to what has been observed in other studies. The increased adhesion of the neutrophils to nylon fibers could be due to the compounds in the herb that may improve the migration of phagocytes such as neutrophils to the foreign body in blood vessels (Guyton and Hall, 2006; Srikumar et al., 2007). The neutrophils represent a multifunctional cell type in innate immunity that contributes to bacterial clearance by recognition, phagocytosis and killing of foreign bodies (Guyton and Hall, 2006; Srikumar et al., 2007), whereas the T and B-lymphocytes are involved and responsible for production of antibodies leading to enhancement of immunity (Guyton and Hall, 2006; Srikumar et al., 2007; Garland-Science, 2008; Soehnlein et al., 2008). The observed increase in the footpad thickness of the Wistar albino rats which is a measure of the DTH as a result of the challenge of the SRBC antigens could be due to some of the compounds found in the herbal extract. The compound might have stimulated the lymphocytes that mediates the delayed type of hypersensitivity or type IV reaction according to the Coomb and Gell (1975) classification (Garland-Science, 2008). The DTH response is a type IV hypersensitivity reaction, which is a direct correlation of cell-mediated immunity. Increase in the DHT indicates that *H. sabdariffa* total crude extracts have a stimulatory effect on lymphocytes and accessory cell types required for the immune reaction (cell-mediated immune response) (Makare et al., 2001; Sharififar et al., 2009). Cell-mediated immunity involves effector mechanisms carried out by T lymphocytes and their products (lymphokines). DTH requires the specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. These in turn increase vascular permeability, induce vasodilatation, microphage accumulation, and activation, promoting increased phagocytic activity and increased concentration of lytic enzymes for more effective killing thus contributing to the observed effect of the cardinal signs of inflammation. When activated, Th_1 cells encounter certain antigens such as SRBCs and they are converted to lymphoblasts (Steven, 2003) and secrete cytokines that induce a localized (defensive) inflammatory reaction called delayed type hypersensitivity (Coombs and Gell, 1975; Garland-Science 2008). The cytokines then attract the scavenger phagocytic cells to the site of reaction hence leading to the type IV delayed hypersensitivity reaction observed in the study when the cells encounter the antigens in form of plant extracts (Marc and Olson, 2009). The observed effect in the delayed hypersensitivity in the rat foot pad could be due to the T-lymphocytes and monocytes and/or macrophages (Garland-Science, 2008; Marc and Olson, 2009). Also the cytotoxic T-cells may have caused direct damage to the foot pad whereas T-helper (TH1) cells may also have increased damage by secreting cytokines that activate cytotoxic T cells that recruit and activate

monocytes and macrophages causing a bulk of tissue damage observed in the study by the increased thickness of the footpad (Garland-Science, 2008; Marc and Olson, 2009). The study has provided evidence for the immunomodulatory activity of *H. sabdariffa* medicinal herb and its continued use by the local communities and traditional herbalist in management of variety of disease conditions.

Conclusion

The total crude leaf extract of *H. sabdariffa* has compounds with immunomodulatory activity on both cell-mediated and humoral-mediated immune responses. It increased red blood cell production and boosted some of the phagocytes. It increased the hemagglutination titers, an indication of boosting the humoral immunity. The results provides evidence for the wide use *H. sabdariffa* as an immune booster in the management of number of disease conditions by both local communities and the traditional health practitioners.

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ABBREVIATIONS

HIV, Human immunodeficiency virus; **CBC,** complete blood count; **HA,** haemagglutinating antibody; **DTH,** delayed-type hypersensitivity; **SRBC,** sheep **RBC,** red blood cells; **EDTA,** ethlenediaminetetracetic acid; **WBC,** white blood cell; **TLC,** total leukocyte cell; **DLC,** differential leukocyte cell; **G-CSF,** granulocytes colony stimulating factors.

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Full Length Research Paper

In vitro study of the desorption kinetic of doxycycline and tetracycline incorporated into collagen controlled released device (CRDs)

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Two demineralized bovine bone membranes after cleaning and cutting into appropriated size, incorporated with doxycycline (DOX) and tetracycline (TET)were evaluated as controlled drug delivery devices and. The complete release time was 96 h (15 days) with a quick release in the first 12 h, decreasing exponentially to zero when it reaches 96 h. The release system fits in a pseudo second order kinetic model allowing the calculation of relevant parameters such as the initial release kinetics of the drug (h) with values of 0.228 µg g^{-1} h^{-1} for DOX and 0.625 µg $g^{-1}h^{-1}$ for TET, and a release rate constant (k) 37.66 g mg^{-1} h^{-1} for DOX and 43.03 g mg^{-1} $h^{-1}$for TET. The amount released by TET is higher than for the DOX in all the periods analyzed, but with the same desorption profile although any additional treatment on the membranes surface has been made. Both systems can be characterized as a controlled release device, due to their effective retention even long time after the start of experiment. Its use incorporated with antimicrobials agents is an important tool as physical barrier in periodontal regeneration to help combat periodontal pathogens and modulate the inflammatory response of the host, limiting tissue destruction.

Key words: Desorption kinetic, doxycycline, tetracycline, membranes, collagen and drug delivery.

INTRODUCTION

Although periodontal disease has a recognized multifactorial nature (Demling et al., 2009; Armitage et al., 1999; Socransky and Haffajee 1993), the dental bacterial biofilm is still considered a primary etiological factor for its establishment and progress (Socransky and Haffajee, 2002; Sbordone and Bortolaia, 2003; Altman et al., 2006). Despite evidence indicating the bacterial specificity of periodontal disease, and the influence of some additional factors in the course of periodontopathies, such as immune response of the host (Slots and Jorgensen, 2002), and environmental factors (Roberts, 2002), periodontal treatment is still based on reducing the supra and subgingival microbiota to levels compatible with the health of the tissues.

Studies demonstrate that although the non-surgical mechanical treatment of scaling and root planning, considered standard treatment, promotes satisfactory results in the control of periodontal inflammation, complete removal of the biofilm and subgingival deposits is rare, particularly in deep sites (Eickholz et al., 2005).

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Thus, antimicrobial agents have been proposed as coadjuvants to conventional mechanical treatment, to help combat periodontal pathogens and modulate the inflammatory response of the host, limiting tissue destruction (Xajigeorgiou et al., 2006).

Antimicrobials may be administered systemically, or by direct application into the periodontal pocket, by using sustained release devices such as Actisite ® (non-degradable tetracycline tape or fiber); Periocline ® (minocycline gel); Elyzol ® (metronidazol gel); Atridox ® (doxycycline powder-liquid system) and Periochip ® (clorhexidine pastilles) (Killoy and Polson ,1998). In recent years, collagen has proven to be an excellent biomaterial, due to its capacity to be chemotaxic to human fibroblasts, its capacity for physiological absorption by the tissues, its hemostatic properties, and because it acts as a support (carrier material) for the cellular growth in the engineering of tissues. Furthermore, collagen is abundant in nature, has an affinity for other synthetic polymers (Li and Wozney, 2001), and is easy to handle in that it can be molded in different forms (Lee et al., 2001).

In odontology, collagen has been used as a material for producing membranes or biological barriers, used in techniques of guided tissue regeneration and guided bone regeneration, due to their biocompatibility and because they can be absorbed (Bunyaratavej and Wang, 2001). Tetracycline (TET) and doxycycline (DOX) are bacteriostatic, with a wide spectrum of action, acting against gram-negative and gram-positive bacteria by specific inhibition of the prokaryotic (bacterian) protein synthesis of ribosome is therefore inhibited in the bacteria, preventing replication and leading to the death of the cell (Pereira-Maia et al., 2010). Besides the anti-bacterial effect, tetracycline and doxycycline have anti-inflammatory and immunosuppressant properties, since they reduce the phagocyte activity of the polymorpho-nuclear leukocytes and the chemotaxis of neutrophils and leukocytes. They also have anti-collagen and anti-lipase action, promoting the repair of conjunctive tissue, which is clinically manifested as increased resistance to probing (Delaissé et al., 2000).

In this study, the authors propose to evaluate *in vitro* the desorption kinetic of two drugs, doxycycline and tetracycline, incorporated into collagen membranes used as controlled release devices (CRD_S) in periodontal treatment.

MATERIALS AND METHODS

Preparation of sustained release devices (SRD) for tetracycline and doxycycline

Collagen membranes with an approximate thickness of 2 µm and area of 2 cm^2 were obtained from decalcification and subsequent lyophilization of bovine cortical bone tissue. They were then immersed, at low temperatures, in a solution containing doxycycline hyclate or tetracycline hydrochloride, for incorporation of drugs into the collagen matrix, giving rise to two controlled release devices: DOX (collagen membrane incorporated with doxycycline) and TET (collagen membrane incorporated with tetracycline). The process of producing these CRDs was carried out at the Biochemistry Department of the Bauru Faculty of Odontology (University of Sao Paulo - Brazil). Both devices were cut in a circular format, with an area of approximately 1 cm^2, and then weighed on 0.001 g precision scales, with mass of 0.012 g for TET and 0.014 g for DOX.

Immersion of the membranes in phosphate buffer

The phosphate buffer solution pH=7.3 ± 0.1 was prepared from the dilution of approximately 2 g sodium hydroxide (NaOH) and approximately 19.8 g of di-hydrogen phosphate of sodium ($NaH_2PO_4.H_2O$) in 1 L of distilled water. Each of the controlled release devices was introduced into a stopped Erlenmeyer flask containing 25 ml of phosphate buffer solution, remaining in a thermostat bath at 36.5 ± 0.1°C throughout the period of the experiment (15 days).

Spectra of absorbance (abs) of buffer solutions containing doxycycline and tetracycline after desorption from the membranes

The buffer solutions containing DOX and TET were analyzed by spectrometry (Shimadzu, model UV 2501 PC) at intervals of 1, 2, 4, 8, 12, 24, 48, 96, 168, 240 and 360 h after the start of the experiment, in processes where the establishment of an equilibrium was avoided, that is, by changing the solvent in contact with the membrane after each measurement. The continuous release of the drug into the solution is therefore presumed. The total absorption spectrum for both samples in solution was run from 190 to 800 nm (ultraviolet and visible) to establish the optimum wavelength of measures. The optimum wavelength common to the two drugs was 275 nm and the phosphate buffer solution was used as reference. Special care was taken with parameters such as concentration, position of the reading in spectrum, and any instrumental interference, in order to avoid significant deviations from the Law of Lambert - Beer. In this case, the absorbance is proportional to the concentration of the solution, and it is possible to determine the amount of doxycycline or tetracycline liberated in the phosphate buffer, based on a calibration curve.

Calibration curves and kinetic parameters to desorption of doxycycline and tetracycline released from devices DOX and TET in phosphate buffer

The correlation between absorbance and concentration of buffer solutions containing TET and DOX was determined empirically, based on a calibration curve constructed with concentrations ranging from $1.86.10^{-5}$ to $24 .10^{-5}$ mol L^{-1} for tetracycline and $6.24.10^{-5}$ to $4.16.10^{-5}$ mol L^{-1} for doxycycline. For this, two standards solutions were prepared with a known concentration of doxycycline (SD), in which 0.050 g of doxycycline hyclate in powder form was diluted in 100 ml of phosphate buffer, and another of tetracycline (ST), in which 0.020 g of tetracycline hydrochloride in powder form was diluted in 100 ml of phosphate buffer. SD and ST were then diluted in various concentrations within the validity of the Lambert-Beer law, and their absorbance was measured.

A linear correlation between absorbance and concentration provides the possibility to determine one of these parameters, knowing the other. Based on this, it was possible to quantify the doxycycline and tetracycline released in phosphate buffer from the devices DOX and TET in 1, 2, 4, 8, 12, 24, 48, 96, 168, 240 and 360 h after the start of experiment (Figure 3).

For desorption kinetic study, model of pseudo second order was

applied, and graphs of t/Q_t of cumulative drug mass released versus time were constructed and desorption rate constant (k) was calculated for DOX and TET, based on the recent results for chemisorption of divalent metal ions onto sphagnum moss peat (Ho, 2006), and another different adsorbate-adsorbent systems (Yuan et al., 2009). Using these data, was calculate also the initial drugs release rate ($h = kQ_e^2$) (Ho, 2006).

RESULTS

The data displayed in Figure 1 shows that there was a release of doxycycline in phosphate buffer, within the environmental conditions proposed by this study. The doxycycline mass observed in the solution after the first hour of the experiment was $2.3.10^{-4}$g. The desorption of the drug proved to be continuous and decreasing in the first 96 h, when the total mass of doxycycline released reached a maximum cumulative of $6.636.10^{-4}$g. Analyzing the data in Figure 1, it is observed that like the doxycycline, there was a continual release of tetracycline in phosphate buffer during the first 96 h of the experiment, reaching a maximum of $10.80.10^{-4}$g. The behavior of the release of the drugs under the experimental conditions adopted is illustrated in this figure, in comparative form. The data also show that tetracycline release into phosphate buffer is higher when compared with the doxycycline, in all the periods analyzed; the mass of tetracycline found in the solution, after the first hour of the experiment, was $3.8.10^{-4}$g.

Figure 2 shows the correlation resulting from the application of the kinetic model pseudo second order (equation1) for the masses cumulative desorbed of drugs released by DOX and TET in phosphate buffer according to equation 2.

$$Q_t = \frac{Q_e^2 kt}{1 + Q_e kt} \quad (1)$$

Where, k is the rate constant of desorption (g/mg min), Q_e the amount of drugs desorbed at equilibrium (mg/g), and Q_t is the amount drugs on the surface of the adsorbent (or liberated from adsorbent surface) at any time, t (mg/g).

$$\frac{t}{Q_t} = \frac{1}{kQ_e^2} + \frac{1}{Q_e}t \quad \text{or} \quad \frac{t}{Q_t} = \frac{1}{h} + \frac{1}{Q_e}t \quad (2)$$

Where, h is the initial desorption rate (mg/g min) as Q_t/t approaches 0. The release profile of the drugs was the same in all the time intervals and a linear correlation was generated (equation 2) after linearization of equation 1. The points between 0 and 96 hours were included, since after this period, drug release is not observed, indicating the end of the desorption process. Initially, between 1 and 12 h, the drug delivery is faster, and after this time, between 12 and 96 h, the process is much slower.

DISCUSSION

The graph of drug delivery using this pseudo second order model linearized shows little difference between both devices (Figure 2). This means that superficial adsorption on this device is limited by the superficial area and desorption process has greatest efficiency during time of dissolution of the drug in the oral liquid. During this time, delivery is fastest. After that, the process became slow, governed by interaction between drugs and membrane surface. In this case, the releasing process can be more controlled if the membrane surface is appropriately worked (Rodrigues et al., 2009). In the case of our membranes, its therapeutic efficiency is limited to four days, from the point of view of drug delivery. The rapid rate of initial delivery of drugs by the devices (between one and twelve hours) has to do with the drug overlay layers on membranes, which features the simple dissolution of the drug, while the slower release between twelve and ninety-six hours was related to interaction between drugs and membrane surface.

The pseudo-second-order expression has been successfully applied to the adsorption of metal ions, dyes, herbicides, oils, and organic substances from aqueous solutions (Ho, 2006) but in this case we used to observe kinetic of drug desorption. By equation (2) we can also estimate the parameter $h = kQ_e^2$, the initial rate of released drug. This parameter is displayed in the Table 1.

DOX and TET, controlled release devices are still in the experimental phase, and are not available for clinical studies involving human beings. Therefore, desorption kinetic of doxycycline and tetracycline was evaluated in this study by means of an *in vitro* experiment. Although it seems more appropriated to expect an first order mathematic model for release of pharmaceuticals from solid matrixes (Ishi, 1996) in this case, the data fit very well to the model proposed. Among all the conditions proposed for the experiment, the continued release is also observed of both drugs in the first 96 h of the study, characterizing DOX and TET as CRD, that is, devices in which desorption of the drug occurs for a period of most of 24 h (Langer and Peppas, 1981; Langer, 1990).

In periodontology, the main purpose of CRD is to release the drug at the site of action for the longest period possible and in inhibitory concentrations for microorganisms periodontopathogenic, without, however, be cytotoxic or promote systemic effects (Marsh, 2003). Periodontal pathogens are susceptible at concentrations of 0.1 to 2.0 $\mu g.ml^{-1}$ of doxycycline and tetracycline (Slots and Rams, 1990). The data obtained in our study show that even the lower concentrations of drugs liberated in phosphate buffer (9.2 $\mu g.ml^{-1}$ from DOX and 15.2 $\mu g.ml^{-1}$ from TET - 1 hour after the start of the experiment; (Table 2), are more than sufficient to inhibit the action of such pathogens. Although not yet esta-blished in the literature is the quantity of doxycycline and tetracycline released locally that are considered cytotoxic; some studies report that very high concentrations of antimicrobial

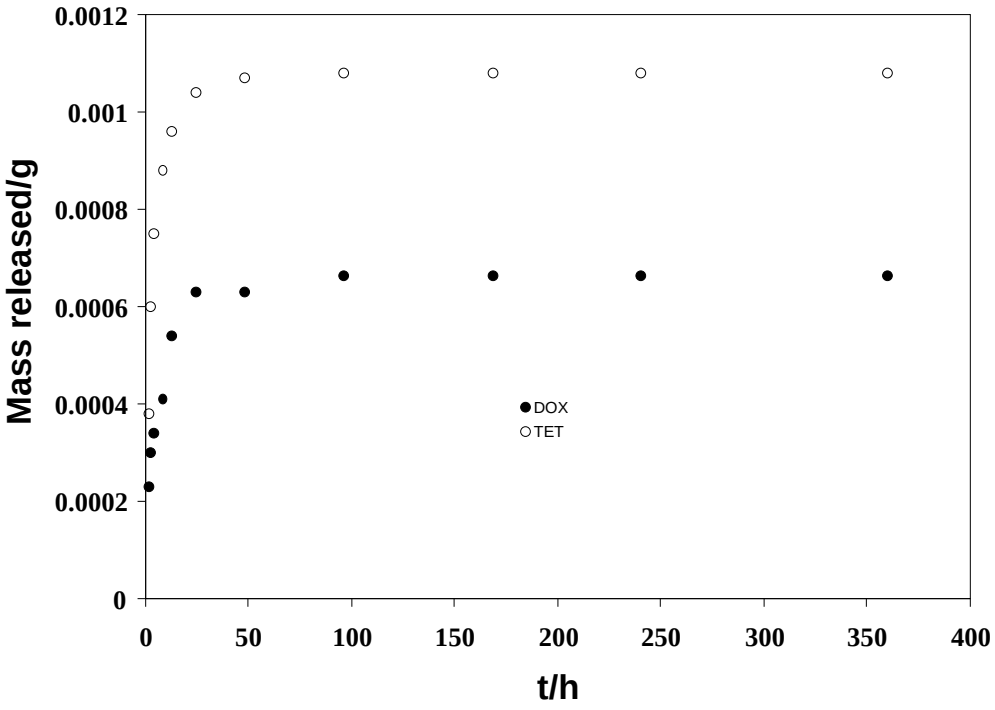
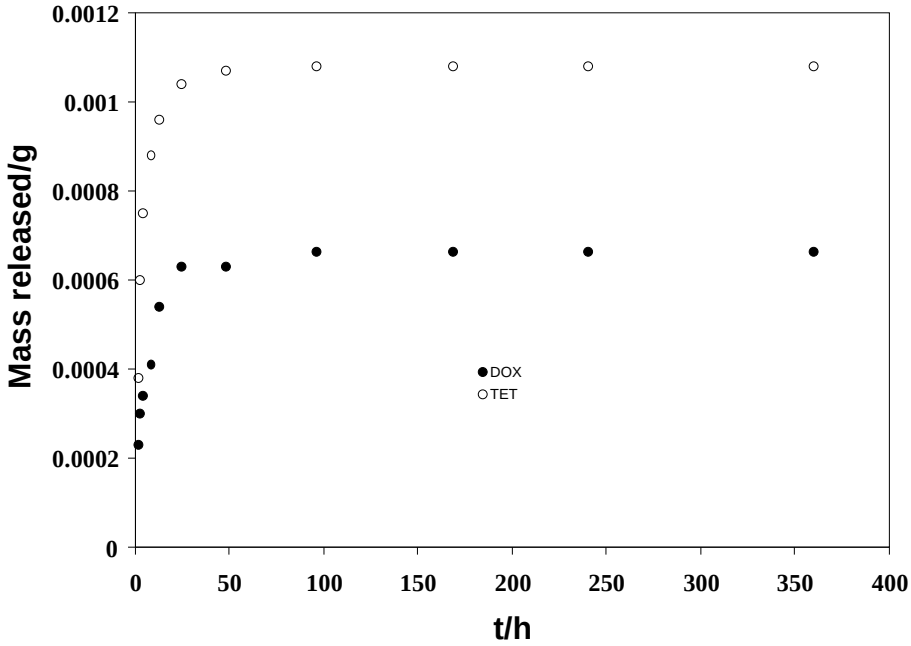


Figure 1. Mass of doxycycline and tetracycline released in phosphate buffer.



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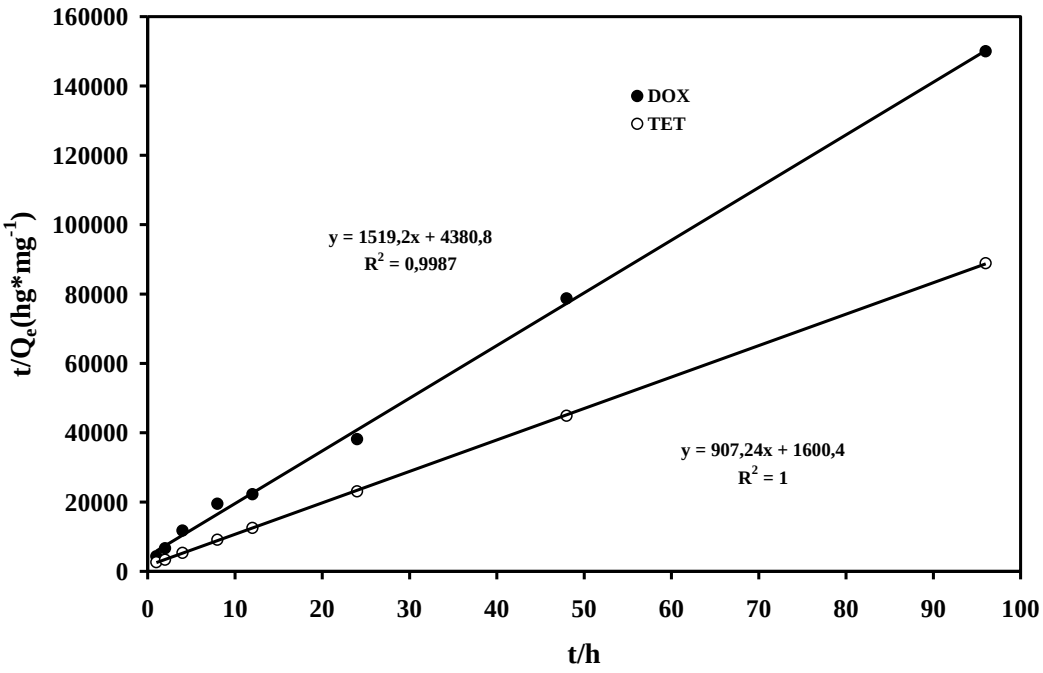

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h [µg $g^{-1}h^{-1}$]	0.228	0.625

Table 2. Concentration of doxycycline and tetracycline in phosphate buffer solutions in evaluated periods.

TIME (h)	C doxycycline [µg.ml^{-1}]	C tetracycline [µg.ml^{-1}]
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much slower, and smaller quantities of drug are continuously released until the liberation process stops completely. The total mass of tetracycline embedded in the membrane was 39% higher than the total mass of doxycycline, and consequently the TET device releases a larger amount of antibiotic in phosphate buffer compared with DOX, in all periods analyzed for these devices. But as can be expected by observing Figures 1 and 2, only a portion of the drug embedded in the membrane should be in direct contact with it, and probably most of the drug is available in overlays. Nevertheless there is one important similarity between the two devices in the process of release of drugs, as can be seen in the cited figures.

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Both devices release doxycycline and tetracycline with a similar profile. The total time of release of the drug by the devices was 96 h. The release mechanism fits in the mathematical model of pseudo second order. The devices have high initial release rate compared to other devices. The TET device releases a higher quantity of antimicrobial in phosphate buffer than the DOX. The membranes of bovine bone collagen (such as DOX and TET) have chemical and mechanical characteristics to be used as devices for controlled delivery of doxycycline and tetracycline in dental and other implants.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

CRDs, Controlled release devices; **TET,** tetracycline; **DOX,** doxycycline; **abs,** absorbance.

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Full Length Research Paper

In vitro study of the desorption kinetic of doxycycline and tetracycline incorporated into collagen controlled released device (CRDs)

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Two demineralized bovine bone membranes after cleaning and cutting into appropriated size, incorporated with doxycycline (DOX) and tetracycline (TET)were evaluated as controlled drug delivery devices and. The complete release time was 96 h (15 days) with a quick release in the first 12 h, decreasing exponentially to zero when it reaches 96 h. The release system fits in a pseudo second order kinetic model allowing the calculation of relevant parameters such as the initial release kinetics of the drug (h) with values of 0.228 µg g^{-1} h^{-1} for DOX and 0.625 µg $g^{-1}h^{-1}$ for TET, and a release rate constant (k) 37.66 g mg^{-1} h^{-1} for DOX and 43.03 g mg^{-1} $h^{-1}$for TET. The amount released by TET is higher than for the DOX in all the periods analyzed, but with the same desorption profile although any additional treatment on the membranes surface has been made. Both systems can be characterized as a controlled release device, due to their effective retention even long time after the start of experiment. Its use incorporated with antimicrobials agents is an important tool as physical barrier in periodontal regeneration to help combat periodontal pathogens and modulate the inflammatory response of the host, limiting tissue destruction.

Key words: Desorption kinetic, doxycycline, tetracycline, membranes, collagen and drug delivery.

INTRODUCTION

Although periodontal disease has a recognized multifactorial nature (Demling et al., 2009; Armitage et al., 1999; Socransky and Haffajee 1993), the dental bacterial biofilm is still considered a primary etiological factor for its establishment and progress (Socransky and Haffajee, 2002; Sbordone and Bortolaia, 2003; Altman et al., 2006). Despite evidence indicating the bacterial specificity of periodontal disease, and the influence of some additional factors in the course of periodontopathies, such as immune response of the host (Slots and Jorgensen, 2002), and environmental factors (Roberts, 2002), periodontal treatment is still based on reducing the supra and subgingival microbiota to levels compatible with the health of the tissues.

Studies demonstrate that although the non-surgical mechanical treatment of scaling and root planning, considered standard treatment, promotes satisfactory results in the control of periodontal inflammation, complete removal of the biofilm and subgingival deposits is rare, particularly in deep sites (Eickholz et al., 2005).

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Thus, antimicrobial agents have been proposed as coadjuvants to conventional mechanical treatment, to help combat periodontal pathogens and modulate the inflammatory response of the host, limiting tissue destruction (Xajigeorgiou et al., 2006).

Antimicrobials may be administered systemically, or by direct application into the periodontal pocket, by using sustained release devices such as Actisite ® (non-degradable tetracycline tape or fiber); Periocline ® (minocycline gel); Elyzol ® (metronidazol gel); Atridox ® (doxycycline powder-liquid system) and Periochip ® (clorhexidine pastilles) (Killoy and Polson ,1998). In recent years, collagen has proven to be an excellent biomaterial, due to its capacity to be chemotaxic to human fibroblasts, its capacity for physiological absorption by the tissues, its hemostatic properties, and because it acts as a support (carrier material) for the cellular growth in the engineering of tissues. Furthermore, collagen is abundant in nature, has an affinity for other synthetic polymers (Li and Wozney, 2001), and is easy to handle in that it can be molded in different forms (Lee et al., 2001).

In odontology, collagen has been used as a material for producing membranes or biological barriers, used in techniques of guided tissue regeneration and guided bone regeneration, due to their biocompatibility and because they can be absorbed (Bunyaratavej and Wang, 2001). Tetracycline (TET) and doxycycline (DOX) are bacteriostatic, with a wide spectrum of action, acting against gram-negative and gram-positive bacteria by specific inhibition of the prokaryotic (bacterian) protein synthesis of ribosome is therefore inhibited in the bacteria, preventing replication and leading to the death of the cell (Pereira-Maia et al., 2010). Besides the anti-bacterial effect, tetracycline and doxycycline have anti-inflammatory and immunosuppressant properties, since they reduce the phagocyte activity of the polymorpho-nuclear leukocytes and the chemotaxis of neutrophils and leukocytes. They also have anti-collagen and anti-lipase action, promoting the repair of conjunctive tissue, which is clinically manifested as increased resistance to probing (Delaissé et al., 2000).

In this study, the authors propose to evaluate *in vitro* the desorption kinetic of two drugs, doxycycline and tetracycline, incorporated into collagen membranes used as controlled release devices (CRD_S) in periodontal treatment.

MATERIALS AND METHODS

Preparation of sustained release devices (SRD) for tetracycline and doxycycline

Collagen membranes with an approximate thickness of 2 µm and area of 2 cm^2 were obtained from decalcification and subsequent lyophilization of bovine cortical bone tissue. They were then immersed, at low temperatures, in a solution containing doxycycline hyclate or tetracycline hydrochloride, for incorporation of drugs into the collagen matrix, giving rise to two controlled release devices: DOX (collagen membrane incorporated with doxycycline) and TET (collagen membrane incorporated with tetracycline). The process of producing these CRDs was carried out at the Biochemistry Department of the Bauru Faculty of Odontology (University of Sao Paulo - Brazil). Both devices were cut in a circular format, with an area of approximately 1 cm^2, and then weighed on 0.001 g precision scales, with mass of 0.012 g for TET and 0.014 g for DOX.

Immersion of the membranes in phosphate buffer

The phosphate buffer solution pH=7.3 ± 0.1 was prepared from the dilution of approximately 2 g sodium hydroxide (NaOH) and approximately 19.8 g of di-hydrogen phosphate of sodium ($NaH_2PO_4.H_2O$) in 1 L of distilled water. Each of the controlled release devices was introduced into a stopped Erlenmeyer flask containing 25 ml of phosphate buffer solution, remaining in a thermostat bath at 36.5 ± 0.1°C throughout the period of the experiment (15 days).

Spectra of absorbance (abs) of buffer solutions containing doxycycline and tetracycline after desorption from the membranes

The buffer solutions containing DOX and TET were analyzed by spectrometry (Shimadzu, model UV 2501 PC) at intervals of 1, 2, 4, 8, 12, 24, 48, 96, 168, 240 and 360 h after the start of the experiment, in processes where the establishment of an equilibrium was avoided, that is, by changing the solvent in contact with the membrane after each measurement. The continuous release of the drug into the solution is therefore presumed. The total absorption spectrum for both samples in solution was run from 190 to 800 nm (ultraviolet and visible) to establish the optimum wavelength of measures. The optimum wavelength common to the two drugs was 275 nm and the phosphate buffer solution was used as reference. Special care was taken with parameters such as concentration, position of the reading in spectrum, and any instrumental interference, in order to avoid significant deviations from the Law of Lambert - Beer. In this case, the absorbance is proportional to the concentration of the solution, and it is possible to determine the amount of doxycycline or tetracycline liberated in the phosphate buffer, based on a calibration curve.

Calibration curves and kinetic parameters to desorption of doxycycline and tetracycline released from devices DOX and TET in phosphate buffer

The correlation between absorbance and concentration of buffer solutions containing TET and DOX was determined empirically, based on a calibration curve constructed with concentrations ranging from $1.86.10^{-5}$ to $24 .10^{-5}$ mol L^{-1} for tetracycline and $6.24.10^{-5}$ to $4.16.10^{-5}$ mol L^{-1} for doxycycline. For this, two standards solutions were prepared with a known concentration of doxycycline (SD), in which 0.050 g of doxycycline hyclate in powder form was diluted in 100 ml of phosphate buffer, and another of tetracycline (ST), in which 0.020 g of tetracycline hydrochloride in powder form was diluted in 100 ml of phosphate buffer. SD and ST were then diluted in various concentrations within the validity of the Lambert-Beer law, and their absorbance was measured.

A linear correlation between absorbance and concentration provides the possibility to determine one of these parameters, knowing the other. Based on this, it was possible to quantify the doxycycline and tetracycline released in phosphate buffer from the devices DOX and TET in 1, 2, 4, 8, 12, 24, 48, 96, 168, 240 and 360 h after the start of experiment (Figure 3).

For desorption kinetic study, model of pseudo second order was

applied, and graphs of t/Q_t of cumulative drug mass released versus time were constructed and desorption rate constant (k) was calculated for DOX and TET, based on the recent results for chemisorption of divalent metal ions onto sphagnum moss peat (Ho, 2006), and another different adsorbate-adsorbent systems (Yuan et al., 2009). Using these data, was calculate also the initial drugs release rate ($h = kQ_e^2$) (Ho, 2006).

RESULTS

The data displayed in Figure 1 shows that there was a release of doxycycline in phosphate buffer, within the environmental conditions proposed by this study. The doxycycline mass observed in the solution after the first hour of the experiment was $2.3.10^{-4}$g. The desorption of the drug proved to be continuous and decreasing in the first 96 h, when the total mass of doxycycline released reached a maximum cumulative of $6.636.10^{-4}$g. Analyzing the data in Figure 1, it is observed that like the doxycycline, there was a continual release of tetracycline in phosphate buffer during the first 96 h of the experiment, reaching a maximum of $10.80.10^{-4}$g. The behavior of the release of the drugs under the experimental conditions adopted is illustrated in this figure, in comparative form. The data also show that tetracycline release into phosphate buffer is higher when compared with the doxycycline, in all the periods analyzed; the mass of tetracycline found in the solution, after the first hour of the experiment, was $3.8.10^{-4}$g.

Figure 2 shows the correlation resulting from the application of the kinetic model pseudo second order (equation1) for the masses cumulative desorbed of drugs released by DOX and TET in phosphate buffer according to equation 2.

$$Q_t = \frac{Q_e^2 kt}{1 + Q_e kt} \quad (1)$$

Where, k is the rate constant of desorption (g/mg min), Q_e the amount of drugs desorbed at equilibrium (mg/g), and Q_t is the amount drugs on the surface of the adsorbent (or liberated from adsorbent surface) at any time, t (mg/g).

$$\frac{t}{Q_t} = \frac{1}{kQ_e^2} + \frac{1}{Q_e}t \quad \text{or} \quad \frac{t}{Q_t} = \frac{1}{h} + \frac{1}{Q_e}t \quad (2)$$

Where, h is the initial desorption rate (mg/g min) as Q_t/t approaches 0. The release profile of the drugs was the same in all the time intervals and a linear correlation was generated (equation 2) after linearization of equation 1. The points between 0 and 96 hours were included, since after this period, drug release is not observed, indicating the end of the desorption process. Initially, between 1 and 12 h, the drug delivery is faster, and after this time, between 12 and 96 h, the process is much slower.

DISCUSSION

The graph of drug delivery using this pseudo second order model linearized shows little difference between both devices (Figure 2). This means that superficial adsorption on this device is limited by the superficial area and desorption process has greatest efficiency during time of dissolution of the drug in the oral liquid. During this time, delivery is fastest. After that, the process became slow, governed by interaction between drugs and membrane surface. In this case, the releasing process can be more controlled if the membrane surface is appropriately worked (Rodrigues et al., 2009). In the case of our membranes, its therapeutic efficiency is limited to four days, from the point of view of drug delivery. The rapid rate of initial delivery of drugs by the devices (between one and twelve hours) has to do with the drug overlay layers on membranes, which features the simple dissolution of the drug, while the slower release between twelve and ninety-six hours was related to interaction between drugs and membrane surface.

The pseudo-second-order expression has been successfully applied to the adsorption of metal ions, dyes, herbicides, oils, and organic substances from aqueous solutions (Ho, 2006) but in this case we used to observe kinetic of drug desorption. By equation (2) we can also estimate the parameter $h = kQ_e^2$, the initial rate of released drug. This parameter is displayed in the Table 1.

DOX and TET, controlled release devices are still in the experimental phase, and are not available for clinical studies involving human beings. Therefore, desorption kinetic of doxycycline and tetracycline was evaluated in this study by means of an *in vitro* experiment. Although it seems more appropriated to expect an first order mathematic model for release of pharmaceuticals from solid matrixes (Ishi, 1996) in this case, the data fit very well to the model proposed. Among all the conditions proposed for the experiment, the continued release is also observed of both drugs in the first 96 h of the study, characterizing DOX and TET as CRD, that is, devices in which desorption of the drug occurs for a period of most of 24 h (Langer and Peppas, 1981; Langer, 1990).

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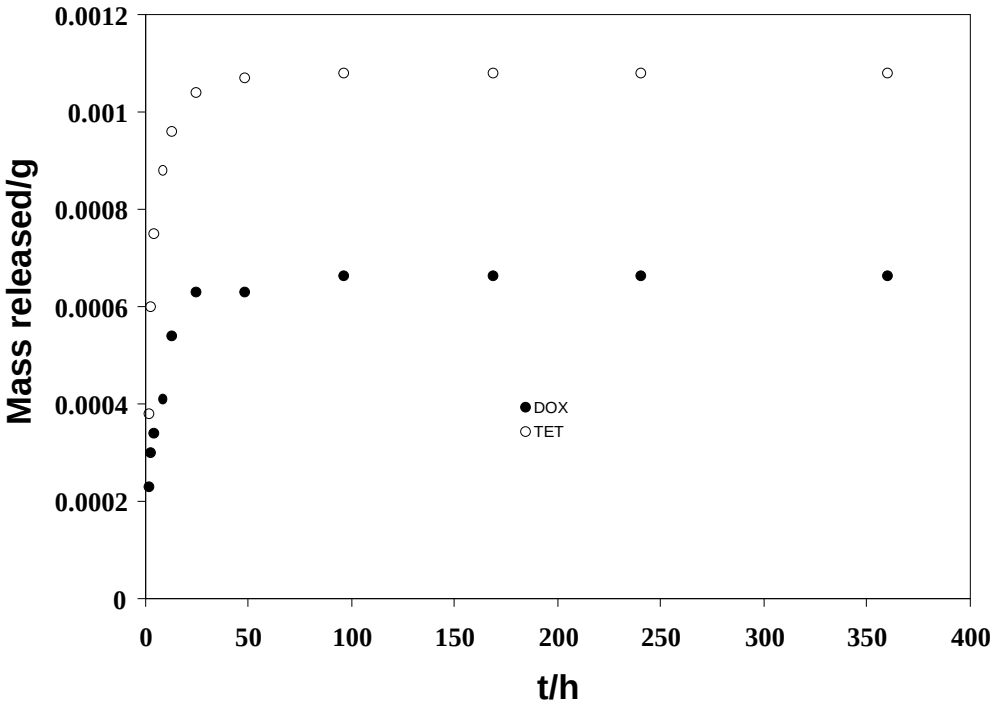

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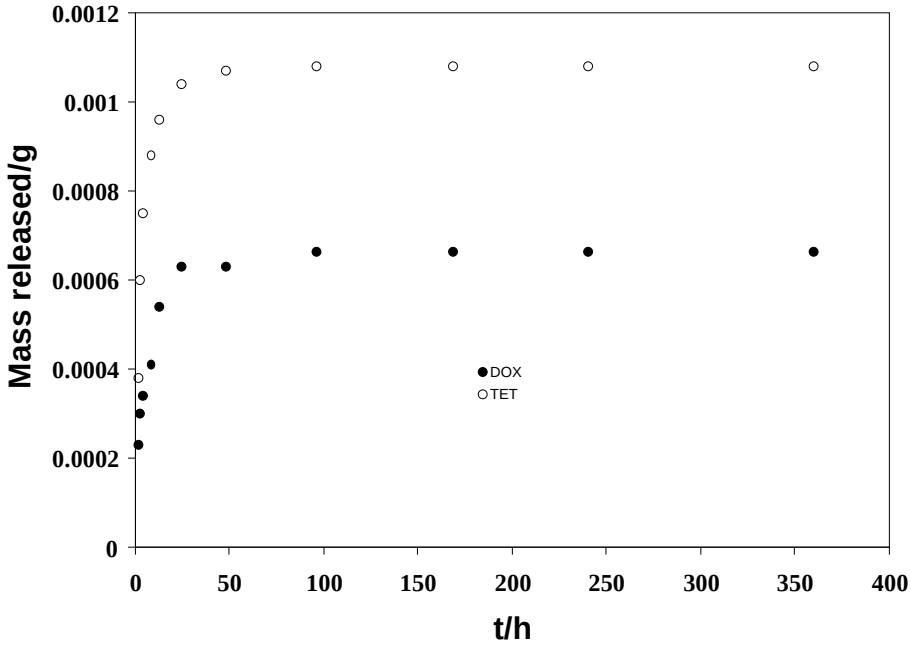

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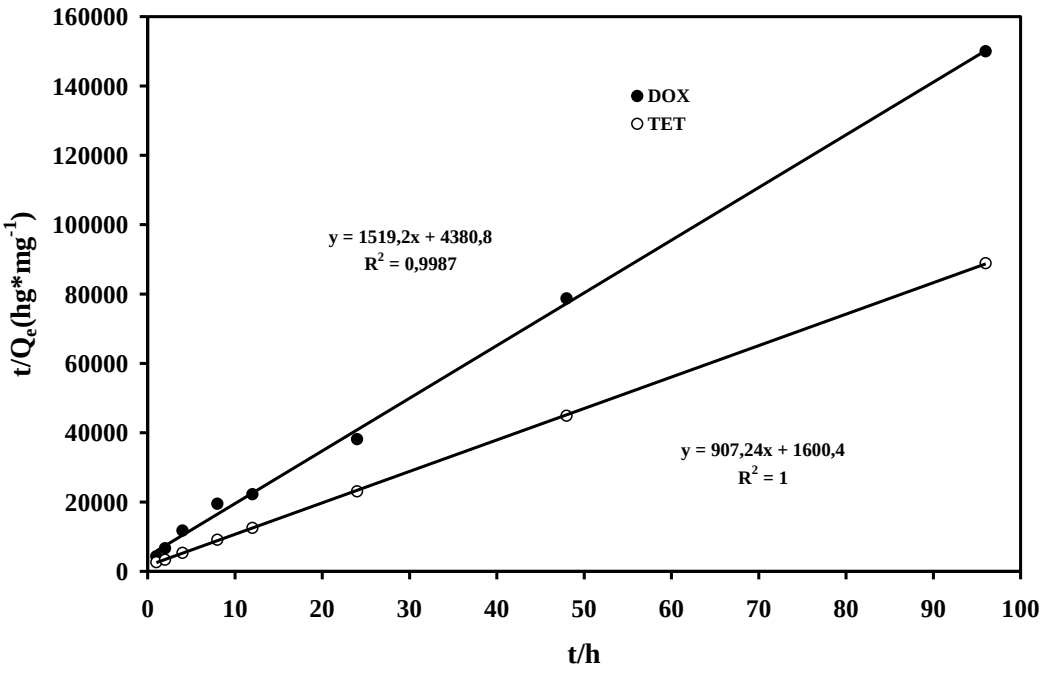

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Both devices release doxycycline and tetracycline with a similar profile. The total time of release of the drug by the devices was 96 h. The release mechanism fits in the mathematical model of pseudo second order. The devices have high initial release rate compared to other devices. The TET device releases a higher quantity of antimicrobial in phosphate buffer than the DOX. The membranes of bovine bone collagen (such as DOX and TET) have chemical and mechanical characteristics to be used as devices for controlled delivery of doxycycline and tetracycline in dental and other implants.

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ABBREVIATIONS

CRDs, Controlled release devices; **TET,** tetracycline; **DOX,** doxycycline; **abs,** absorbance.

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Full Length Research Paper

Preparation and characterization of orodispersible tablets of Meclizine Hydrochloride by wet granulation method

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The aim of this research is to prepare orodispersible tablets of Meclizine Hydrochloride (MHCl). MHCl is used to treat or prevent nausea, vomiting, and dizziness. Wet granulation method was used to prepare the orodispersible tablets of MHCl using different super disintegrant; crospovidone (CP), sodium starch glycolate (SSG), croscarmellose sodium (CCS), and microcrystalline cellulose (MCC) at different concentrations. Camphor and ammonium carbonate were also incorporated in preparation as subliming agents. Co processing of super disintegrant with MCC is a new approach to increase disintegration rate which we tried. The prepared tablets were evaluated for weight variation, content uniformity, hardness, disintegration time, and friability of tablets. All the formulations showed low weight variation with *in-vitro* disintegration time less than 71 s. The drug content of all the formulations was within the acceptable limits. Crospovidone shows the shortest disintegration time among super disintegrants while use of subliming agent produced friable tablets. Although use of combination of super disintegrant with MCC decreases disintegration time, the use of co processed super disintegrant (10%CP with 20% MCC) provides the optimum properties of orodispersible tablets (F11). Stability study of selected formula showed no significant changes in tablet properties.

Key words: Meclizine hydrochloride, wet granulation method, subliming agent, orodispersible tablet, co process.

INTRODUCTION

The disadvantages of conventional solid oral dosage forms is the necessity of water to enhance swallowing the dosage forms specially for certain groups of patients as geriatrics, pediatrics, and unconscious patients and during travelling (Venkatal et al., 2009). The continuous developing of dosage forms in the last years reveals new one which the ability to overcome the limitation of normal dosage form since it is required water for help in swallowing. The definition of orodispersible tablet in European Pharmacopoeia as “A tablet that to be placed in the mouth where it disperses rapidly before swallowing in less than three minutes” (Kamal et al., 2010). The (ODT) technology has been recently approved by United States Pharmacopoeia (USP), Centre for Drug Evaluation and Research (CDER). United States Food and Drug Administration (FDA) defined orally disintegrating tablet as “A solid dosage form containing medicinal substance or active ingredient which disintegrates rapidly usually within a matter of seconds when placed upon the tongue” (Bhupendra et al., 2010). The ODT is also known as fast melting, fast dispersing, rapid dissolve, rapid melt, and/or quick disintegrating tablet (Yourong et al., 2004). When

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an ODT is placed in the oral cavity, saliva quickly penetrates into the pores causing rapid disintegration. ODTs are useful in patients, such as developmentally disabled or who may face difficulty in swallowing conventional tablets or capsules and liquid orals or syrup, leading to ineffective therapy, with persistent nausea, sudden episodes of allergic attacks, or coughing for those who have an active life style (Kaushik et al., 2004; Chue et al., 2004; Shu et al., 2002; Seager et al., 1998; Gohel et al., 2004).

ODTs are also applicable when local action in the mouth is desirable such as local anesthetic for tooth-aches, oral ulcers, cold sores, or teething, and to deliver sustained release multiparticulate system to those who cannot swallow intact sustained action tablets/capsules (Chang et al., 2000; Shimizu et al., 2003).

Meclizine HCl, an oral antiemetic, is a white, slightly yellowish, crystalline powder which has a slight odor and is tasteless. Meclizine HCl is an antihistamine which shows slower onset and longer duration of action (24 h) than most other antihistamines used for motion sickness. Meclizine hydrochloride is an antiemetic drug indicated in prophylactic treatment and management of nausea and vomiting, and dizziness associated with motion sickness (Moffat et al., 2004). The aim of the present work was to evaluate the potential of super disintegrant and subliming agent in production of Meclizine HCl ODTs with acceptable mechanical strength and disintegration time.

EXPERIMENTAL

Materials

Meclizine HCl powder was purchased from Oceanic Pharmachem, India, Crospovidone (CP), and Croscarmellose Sodium (CCS) were purchased from 3B Pharmaceutical (Wuhan) international Co. Ltd, China. Magnesium stearate, Mannitol, Ammonium carbonate were purchased from Riedel-De-Haen AG seelze, Germany. Camphor was purchased from Evans Medical Ltd, Liverpool, England. All other materials were of analytical grade.

Methods

Formulation of orodispersible tablets of Meclizine hydrochloride

All the ingredients (Except Lubricants and glidant) were passed through mesh No.44 meshes separately, then weighed and mixed in geometrical order for about 10 min. A sufficient quantity of freshly prepared starch mucilage of concentration 5% w/v was added to produce a wet coherent mass (Alebiowu et al., 2002). There after the wet mass was passed through mesh No.14 and the wet granules were dried at 50°C to constant weight in a hot air oven. Then the dried granules were passed through sieve mesh No 18. Then lubricants and glidant were added to the mixture and mixed for about 2 min. Finally an amount of the blend was compressed into tablets of 200 mg using 8 mm round flat punches using sixteen station rotary tablet machine (Vanguard Pharmaceutical, USA). Sublimation was performed from tablets contain subliming agents at 60ºC until a constant tablet weight was achieved (Koizumi et al., 1997). A minimum of 50 tablets were prepared for each batch.

Preparation of co-processed super disintegrant

The co-processed super disintegrant was prepared by solvent evaporation method (Table 1). A blend of CP and MCC (in the ratio of 1: 2) was added to 10 ml of ethanol. The contents of the beaker (250 ml capacity) were mixed thoroughly and stirring was continued till most of ethanol evaporated. The wet coherent mass was granulated through mesh No. 44. The wet granules were dried in a hot air oven at 60°C for 20 min. The dried granules were sifted through mesh No. 44 and stored in airtight container till further use (Gohel et al., 2007).

Pre compression parameters

Angle of repose

Angle of repose was determined using funnel method (British Pharmacopoeia, 2007). The granules were poured through funnel that can be raised vertically until a maximum cone height (h) was obtained. Radius of the heap (r) was measured and angle of repose was calculated using the formula:

$\tan \theta = h/r$

where, θ is the angle of repose, h is height of pile; r is radius of the base of pile.

Compressibility (Carr's) index

An accurate weight of formula granules was poured in to a volumetric cylinder to occupy a volume ($V_\circ$) and then subjected to a standard tapping procedure on to a solid surface until a constant volume was achieved (V_f). The Carr's index was calculated using following equation (British Pharmacopoeia, 2007).

Compressibility Index = $100x\ (V_0-V_f)/V_0$

Evaluation of the prepared orodispersible Meclizine hydrochloride tablets

Weight variation

Randomly, twenty tablets were selected after compression and the mean weight was determined. None of the tablets deviated from the average weight by more than ±7.5%.

Uniformity of content

One tablet of the prepared formula was placed in 100 ml volumetric flask, 50 ml of 0.01 N HCL was added, shaked by mechanical means for 30 min, the dilute acid added to volume, and filtered sample was analyzed for Meclizine hydrochloride in the tablet spectrophotometrically using UV-Visible spectrophotometer (Silverstein et al., 1991). The requirement for this test is met if the amount of drug in each of the ten tablets lies within the range of (85-115%) of the label claim.

Table 1. Different formulas used in preparation of orodispersible tablets of MHCl

Ingredients (mg)	Formulation code										
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11*
MHCL	25	25	25	25	25	25	25	25	25	25	25
Crospovidone	10	20					20	20	20	20	20
SCC			10	20							
SSG					10	20					
Camphor							20				
Ammonium bicarbonate								20			
MCC									20	40	40
Starch mucilage (5%w/v)	Q.S	Q.S	Q.S	Q.S	Q.S	Q.S	Q.S	Q.S	Q.S	Q.S	Q.S
Mg Stearate	3	3	3	3	3	3	3	3	3	3	3
Talc	3	3	3	3	3	3	3	3	3	3	3
Cab-O-Sil	2	2	2	2	2	2	2	2	2	2	2
Lactose up to	200	200	200	200	200	200	200	200	200	200	200

F11, coprocess of CP and MCC.

Wetting time

A piece of tissue paper (12 cm × 10.75 cm) folded twice was placed in a Petri dish (Internal Diameter=9 cm) containing 10 ml of buffer solution simulating saliva pH 6.8 and amaranth. A tablet was placed on the paper and the time taken for complete wetting was noted. Three tablets from each formulation were randomly selected and the average wetting time was recorded (Hisakadzu et al., 2002).

Hardness

The crushing strength of the tablets was measured using a Monsanto hardness tester. Three tablets from each formulation batch were tested randomly and the average reading ± SD was recorded.

Friability

Twenty tablets were weighed and placed in a Roche friabilator and the equipment was rotated at 25 rpm for 4 min. The tablets were taken out, de dusted and reweighed. The percentage friability of the tablets was calculated using following equation (Rahul et al., 2009).

Percent friability = (Initial weight-Final weight)/ Initial weight × 100)

In vitro disintegration time

The disintegration time was defined as the time in seconds taken for complete disintegration of the tablet with no palpable mass remaining in the apparatus was measure in second using artificial saliva as disintegration medium.

Six tablets were placed individually in each tube of disintegration test apparatus. The values reported are mean ± standard deviation (Mohsin et al., 2010).

In vitro drug dissolution test

In vitro dissolution studies were performed only for the optimum formula and Meclizine (reference tablet, 25 mg) by using type I (Basket) dissolution apparatus (10ST+ G.B Caleva Ltd ,Dorset ,England), at 100 rpm, and 900 ml of 0.01 N HCL was used as a dissolution medium. Temperature of dissolution medium was maintained at 37 ± 0.5°C. Five millimeter aliquot of the dissolution medium was withdrawn at specific time intervals and it was filtered. Absorption of filtered solution was read by UV- visible spectrophotometer (UV-1650PC- Shimadzu, Japan) at λ_{max}= 232 nm and drug content was determined from a standard calibration curve (United States Pharmacopoeia, 2007). The mean of three determinations was used ± SD.

The percent of drug dissolved in 15 min was considered for comparing the dissolution results.

Statistical analysis

The mean ± standard deviation of the experiments results were analyzed using one way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Meclizine HCl tablets were prepared by wet granulation method. Eleven formulations were prepared using two different subliming agents alone or with three different super disinegrants. Each super disinegrant was used in two different concentrations (5 and 10%). All batches of the tablets were evaluated for various pre and post compression parameters. Table 2 shows the data obtained from the pre compression evaluation of tablets which includes angle of repose, and Carr's index while post compression parameters such as hardness, friability, drug content, wetting time, and disintegration time were evaluated.

The results of flowability studies of the granules reveals acceptable flowability for tablet production represented by the angle of repose and Carr's index values listed in Table 2.

Table 2. Pre-compression parameters of MHCl orodispersible formulas.

Formula no.	Angle of repose (˚)	Carr's index	Flow character
F1	31.76 ±0.94	17.53 ±0.97	Good and Fair
F2	26.33 ±0.76	16.71 ±1.20	Excellent and Fair
F3	32.42 ±0.97	18.54 ±1.80	Good and Fair
F4	31.57 ±0.99	16.55 ±1.11	Good and Fair
F5	32.66 ±1.24	16.48 ±0.75	Good and Fair
F6	31.27 ±1.34	18.23 ±0.98	Good and Fair
F7	31.81 ±1.40	19.48 ±0.65	Good and Fair
F8	20.43 ±1.56	17.23 ±0.64	Excellent and Fair
F9	24.17 ±0.96	19.78 ±1.21	Excellent and Fair
F10	26.35 ±1.23	18.66 ±1.47	Excellent and Fair
F11	28.47 ±1.47	18.76 ±0.83	Excellent and Fair

Table 3. Post-compression parameters of prepared MHCl orodispersible tablets.

Formula no.	*In vitro* DT (s)	Wetting time (s)	Hardness (kg/cm^2)	Friability
F1	31±1.72	28±0.33	6.1±0.76	0.64
F2	26±1.21	24±0.81	6.8±0.78	0.53
F3	47±1.92	63±0.97	3.9±0.64	0.66
F4	58±1.43	75±1.12	5.1±0.61	0.58
F5	67±1.78	99±0.98	4.8±0.54	0.92
F6	71±1.45	107±1.7	3.9±0.33	0.69
F7	28±1.45	25±0.74	7.0±0.93	1.10
F8	25±0.76	28±1.45	2.5±0.61	1.74
F9	23±1.43	31±0.75	3.9±0.77	0.79
F10	22±0.43	25±0.66	4.1±1.54	0.71
F11	20±0.45	23±0.43	4.2±0.33	0.87

The post compression parameters of all prepared tablets are reported in Table 3 indicate that the hardness in the range 3.9 to 7 kg/cm^2 which is appropriate except formula that contain ammonium carbonate as subliming agent shows low hardness (2.5 kg/cm^2). The loss in total weight of the tablets due to friability was in the range of 0.53 to 0.92% except formulas contain subliming agent (F7 and F8) produces friable tablets which mainly due to high porosity of tablets. The drug content in different formulation was highly uniform and in the range of 98.13 to 100.74%.

Wetting time is an important parameter shows the efficiency of super disintegrant regarding the swelling in presence of water was found to be 23 to 107 s. The results of *in vitro* disintegration time indicate that formulas which contain only super disintegrant shows efficiency in the following order; crospovidone > croscaramellose sodium >sodium starch glycolate, with superiority of 10% over 5% super disintegrant concentration for the three types and 10% is the optimum percent for good disintegration.

Although formulations used with subliming agent shows shorter disintegration time than that with super disintegrant, but the tablets produced were highly friable. Combination of best super disintegrant (CP) with MCC reduce the disintegration time and improves mechanical properties, however new approach of co processing of the super disintegrant (CP) with MCC (F11) produces the shortest disintegration time since granules of co-processed super disintegrant possesses greater density and its particles are closer to each other than the physical mixture therefore after water uptake a greater hydrostatic pressure formed that strongly repels the particle from one another and ultimately results in quicker tablet disintegration. The results are in agreement with those of Shirsand et al. (2010). Formula (F11) was selected as best formula thus subjected for dissolution studies in comparison to conventional formula as shown in Figure 1. The results of dissolution study (Figure 1) show that the release of MHCl from selected formula and conventional tablet shows release of 100 and 49.32% respectively at 15 min which consequently indicates that enhancement of release was obtained for the selected formula.

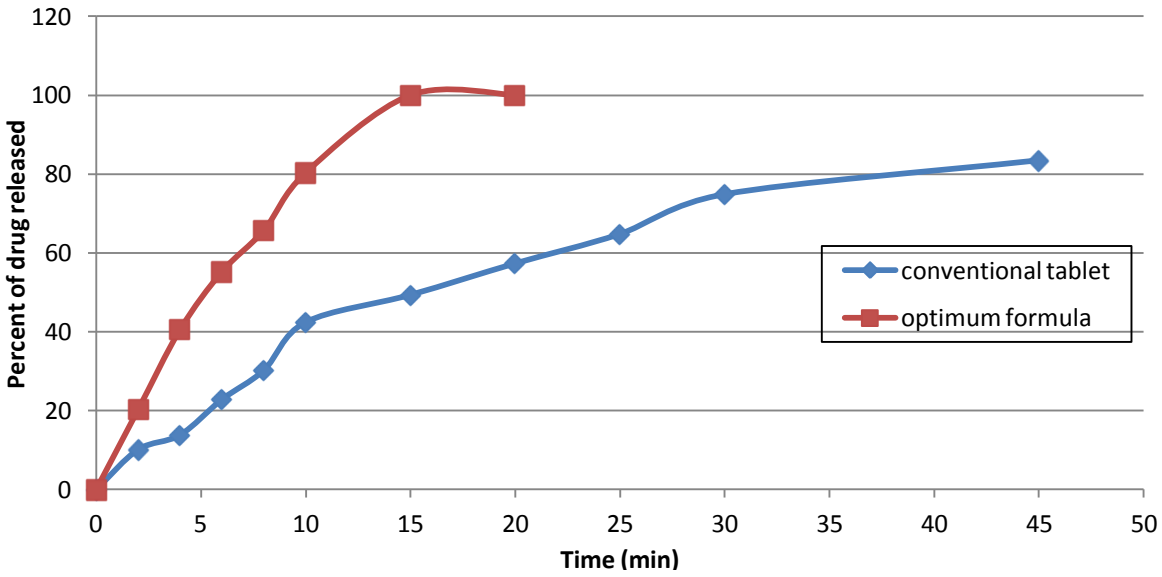

Figure 1. Dissolution profile of MHCl from selected formula and conventional tablet.

Conclusion

Formulation of ODTs of MHCl by wet granulation method using coprocessed CP 10% with MCC exhibited fast disintegration time with good mechanical properties.

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Full Length Research Paper

Lovastatin production using *Pleurotus ostreatus* and its medicinal properties analysis by docking

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Lovastatin is an anti-lipidemic drug produced by various filamentous fungi as a secondary metabolite. In our study, *Pleurotus ostreatus* was used in the production of lovastatin by solid state fermentation process. *P. ostreatus* was grown on different substrate (wheat bran, rice bran, rice straw, sugarcane bagasse), from which the maximum yield was obtained when wheat bran was used as a substrate in solid substrate fermentation. Further, wheat bran was used as a substrate and was grown on different temperatures (25, 28, 32 and 35°C), in which a maximum yield was obtained at 28°C. To determine its anti-lipidemic property and other medicinal properties like anti cancer, Alzheimer's disease a docking study was done using Auto dock Vina, in which various proteins responsible for the disease are targeted and studied. The docking studies prove that lovastatin can effectively help in the treatment of various diseases by effectively binding to various proteins which are responsible for cancer, apoptosis and Alzheimer's.

Key words: Lovastatin, fermentation, wheat bran, hydroxymethylglutaryl CoA reductases, Alzheimer disease.

INTRODUCTION

Lovastatin is a first known drug in statin family which was discovered in 1970s (Endo, 1976). It was initially produced from *Aspergillus terrus* (Endo, 1976). Various other organisms like *Pleurotus ostreatus* (Samiee et al., 2003), a marine actinomycetes (Srinu et al., 2010), *Aspergillus parasiticus*, *Accremonium chrysogenum* (Endo, 1992), *Monascus pirpureus* (Danuri, 2008) also has the ability to produce lovastatin. Lovastatin has many medicinal properties; therapeutically used as an anti-lipidemic for the reduction of cholesterol. The blood cholesterol level reduced by inhibiting the HMG- Co A reductase enzyme (Tobert, 1987), is an important enzyme in formation of cholesterol from acetyl Co A. Various other statin drugs were synthesized from lovastatin by semi-synthetic process. A huge study is being done for the production of lovastatin through solid state fermentation process as a low cost substrates like wheat bran, rice straw can be used, and also the water and power consumption are very less when compared with submerged fermentation process (Rajput and Raj, 2009). The quantity of lovastatin produced from the fermentation can be quantified by microbial assay using *Candida albicans* as lovastatin has an inhibitory effects on the mycelium fungus, which is based on the anti fungal property (Kumar et al. 2000). Long term use of lovastatin has shown to exhibit anti-cancer properties in human and also have shown to cure Alzheimer's disease in rats (Eckert et al, 2005). Even though many plants such as *Zanthoxylum armatum*, *Origanum majorana* L., *Peganum harmala* L, and *Salvia officinalis* L are known to show a wide range of medicinal properties, a huge study need to be done to find its exact mechanism of action and also needs more formulation to be used as a medicine (Barakat et al., (2013); Steenkamp et al., 2013 Samy et al., 2013). Lovastatin can be used as a drug for various diseases by studying its mechanism of action with various proteins responsible for inducing of disease. A docking study was done with various proteins,

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interacting with lovastatin to know its binding over other proteins and to find its mechanism of action against various diseases. Glycogen synthase kinase-3β, thymedylate synthase, TGF-β receptor type I, focal adhesion kinase 1, dihydrofolate reductase are few proteins which were targeted using chemotherapy to cure cancer in humans. Other proteins such as caspase-3, cyclin-dependent protein kinase-2, cyclin-dependent protein kinase-5, cyclin-dependent protein kinase-7, cyclin-dependent protein kinase-9, known as apoptosis protein, whose malfunction may lead to cancer, were also targeted.

In our study, various solid substrates (wheat bran, rice bran, rice straw and sugarcane bagasse) were screened to find the maximum yield and optimized temperature of lovastatin. Also, its medicinal properties were studied on various cancer and apoptosis proteins. Proteins responsible for Alzheimer's were taken and the docking study was done with lovastatin to know the effectiveness of the drug.

MATERIALS AND METHODS

Materials

All chemicals were of analytical grade obtained from Central drug house (CDH), Chennai, India including potato dextrose agar (PDA), $MgSO_4.7H2O$, $(NH_4)_2HPO_4$, NaCl. Various agricultural wastes such as wheat bran, rice bran, rice straw, sugarcane bagasse, oats meal, coconut shells, saw dust, corn, soya bean, chickpea shells were purchased from local market of Tamil Nadu, India. Lovastatin tablets was purchased from pharmacy in Chennai, Tamil Nadu, India and used as standard.

Microorganisms

The fungus, *P. ostreatus* was obtained from University of Madras, Chennai, Tamil Nadu. The stock cultures were maintained in PDA at 4°C and were subcultured for every 15 days with PDA. The sub cultures were grown in potato dextrose broth for 4 days and used as inoculum in solid state fermentation.

Screening of different substrates for lovastatin production

P. ostreatus was grown on different solid substrates (wheat bran, rice bran, rice straw, sugarcane bagasse). 10 g of each solid substrates was taken separately in Petri plates and was moistened with distilled water and steam sterilized at 121°C for 15 min. The medium was cooled and inoculated with a four day old *P. ostreatus* culture. The culture flasks were then maintained at 25°C for 8 days. Further, the lovastatin was screened using *C. albicans*.

Temperature optimization on lovastatin production

A solid state fermentation was carried out using wheat bran as a solid substrate, where 5 g of the substrate was moistened with distilled water containing $MgSO_4.7H_2O$ (0.15g/l), $(NH_4)_2HPO_4$ (0.25g/l), NaCl (1g/l) and steam sterilized at 121°C for 15 min. They were inoculated with *P. ostreatus* and maintained at different temperatures (25, 28, 32, 35 and 40°C), the culture was extracted on day seven and quantified. The experiments were conducted on duplicates and the analyses were performed in triplicates. The data were statistically analyzed and standard error bars were obtained.

Extraction of lovastatin

P. ostreatus was incubated for eight days at 28°C. Later, plates were taken and dried at 60°C in hot air oven. From the dried culture, 1 g of the culture was taken in a test tube, to which 10 ml of ethylacetate was added. This was vortexed for 15 min and stored at cold condition for 1 h, centrifuged at 3000 g for 15 min for layer separation. The supernatant was collected and bioassay was carried at using *C. albicans* to find the concentration of lovastatin present in the culture extract as explained below.

Bioassay with *Candida albicans*

C. albicans was grown on PDA for 12 h at 28°C. *C. albicans* was subcultured on fresh PDA plates at a concentration of 7×10^3 cells/ml and grown at 28°C. Fifty micro liters of the extracts were taken and transferred to 6 mm paper disk and placed on 90 mm petri plate containing *C. albicans*. The spacing between the control and the lovastatin were adjusted to be 15 mm. Positive and negative controls were prepared by impregnating the paper with 50 µl of known concentration of lovastatin standard and ethylacetate, respectively. The plates were incubated for 12 h and zone of inhibition was recorded. A large diameter of the inhibition zone indicated a high titre of lovastatin (Vilches Ferrón et al., 2005).

Docking of lovastatin using protein molecules

Autodock was carried out for lovastatin drug to study the various medicinal properties. Autodock vina version 1.1.2, 2012. (Trott and Olson 2010) was used for the docking of lovastatin with various protein molecules. They were visualized using Molegro molecular (René Thomsen and Mikael H. Christensen, 2006.) viewer tool.

RESULTS

Screening of solid substrates for lovastatin production

Different solid substrates (wheat bran, rice bran, rice straw, sugarcane bagasse) were used to grow *P. ostreatus* to find the best solid substrate for maximum yield of lovastatin. Table 1 shows the results obtained for various substrates.

Effect of temperature on production of lovastatin

Various temperatures (25, 28, 32 and 35°C) was maintained in *P. ostreatus* culture and their effect on lovastatin was determined on day seven. Figure 1 shows the yield of lovastatin on various temperatures.

Study of various medicinal properties of lovastatin using autodock vina

Lovastatin was docked with various proteins using

Table 1. Yield of lovastatin using various solid substrates

Solid substrates	Inhibition zone (mm)	Concentration of lovastatin(µg/ml)
Wheat bran	7	115
Rice straw	2.4	39.5
Rice bran	3.1	51
Sugarcane bagasse	1.9	31.1

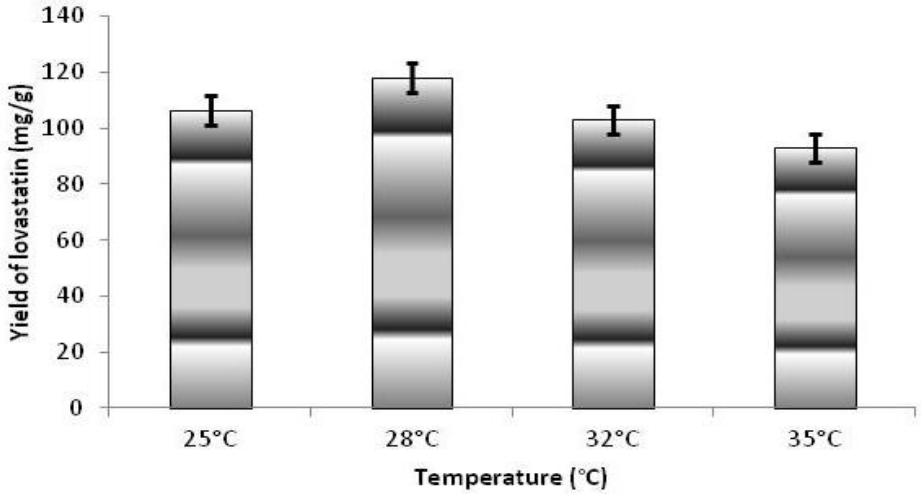

Figure 1. Effect of temperature on lovastatin production on wheat bran as a substrate.

Table 2. Active sites of proteins related to Alzhemier's disease.

S/N	Protein	PDB ID	Binding affinity	Binding sites
1	Tau Protein	1J1B	-8.1	Asn 564
				Pro 636
				Arg 641
				Tyr 634
2	TNF-α	2AZ5	-7.9	Tyr 552
				Gly 382
3	TNF-β	1TNR	-7.6	Oxygen

autodock vina to study its medicinal properties, site of action and its mechanism of binding over proteins for the treatment of various disorders.

Proteins responsible for Alzheimer's disease

Alzheimer's is a disease where the brain loses its functions (memory and thinking), as time progress, it leads to death. Various proteins like tumor necrosis factor (TNF), Tau proteins are responsible for the cause of the disease (Gong et al, 2010). Lovastatin has shown some effects on Alzheimer's disease; a study was carried out to find the interaction between various proteins (Table 2) that are responsible for Alzheimer's disease and lovastatin.

Proteins responsible for cancer proteins

Cancer is the uncontrolled growth of cells; there are many cancers which are caused due to various reasons. Many proteins are responsible for the cause of cancer; an interaction study was done between various cancer protein and lovastatin drug molecule, to study the property of lovastatin on cancer cells (Xia et al., 2001). Total of five proteins were docked individually with lovastatin and their interaction and site of action on

Table 3. Active region of cancer proteins.

S/N	Protein	PDB ID	Binding affinity	Binding sites
1	Glycogen synthase kinase-3β	1UV5	-8.1	Lys 85
				Arg 141
2	Thymidylate Synthase	1JU6	-7.7	Met 881
				Arg 650
3	TGF-beta receptor type I	1PY5	-9.5	Lys 213
				Ser 287
4	Focal adhesion kinase 1	3BZ3	-7.1	Thr 474
				Arg 569
5	Dihydrofolate reductase	3GI2	-6.7	Ser 118
				Thr 56

Table 4. Active sites of apoptosis protein.

S/N	Protein	PDB ID	Binding Affinity	Binding sites
1	Caspase-3	1GFW	-7.7	Ser 209
				Ser 65
				Arg 207
2	Cyclin-dependent protein kinase-7	1UA2	-7.6	Leu 257
				His 258
3	Cyclin-dependent protein kinase-5	1UNH	-8.2	Asn 408
				Asn 421
4	Cyclin-dependent protein kinase-2	2UZO	-8.4	His 42
				Gly 122
5	Cyclin-dependent protein kinase-9	3BLR	-8.7	Glu 107
				Asp 109

proteins (Table 3) were studied.

Proteins responsible for apoptosis

Apoptosis is a process of programmed cell death, which occurs in multi-cellular organism. The process helps in regulation of cell cycle, repair of cell damage and also mitochondrial regulation. Malfunction of apoptosis may lead to several problems depending on its property, where insufficient apoptosis may lead to cancer, auto immune disorder and several viral infections, while excessive apoptosis may leads to several neurodegene-rative problems and also cause myocardial infarction. Lovastatin shows inhibition over apoptosis (Tandon et al., 2005), so a docking was carried out for six apoptosis protein against lovastatin as a ligand (Table 4), to study the efficiency of lovastatin for the treatment of various diseases.

DISCUSSION

A maximum yield of 115 µg/ml of lovastatin was obtained when wheat bran was used as a solid substrate. Followed by rice bran, rice straw, sugarcane bagasse gave a yield of 51, 39.5 and 31.5 µg/ml, respectively. The maximum yield of lovastatin was obtained from wheat bran, which may be due to easy absorption of nutrients from them and also due to maintenance of moisture content in the substrate. The temperature optimization which increases the yield at an optimum condition of 28°C is in accordance with the results obtained by Shami et al. (2007). A considerable yield of 106.5 and 103 mg/g lovastatin was obtained at temperature 25 and 32°C, respectively. A low yield of lovastatin was obtained at other temperatures (35 and 40°C). This may be due to the incompatibility of the organism to grow at these temperatures and also due to raise in temperature, there is a great loss in the moisture content which affects the growth of the organism which in turn reduced the yield of lovastatin.

Inhibition of lovastatin with HMG-Co A reductase (PDB ID: 1DQ9)

A docking study was carried out to study the effect of

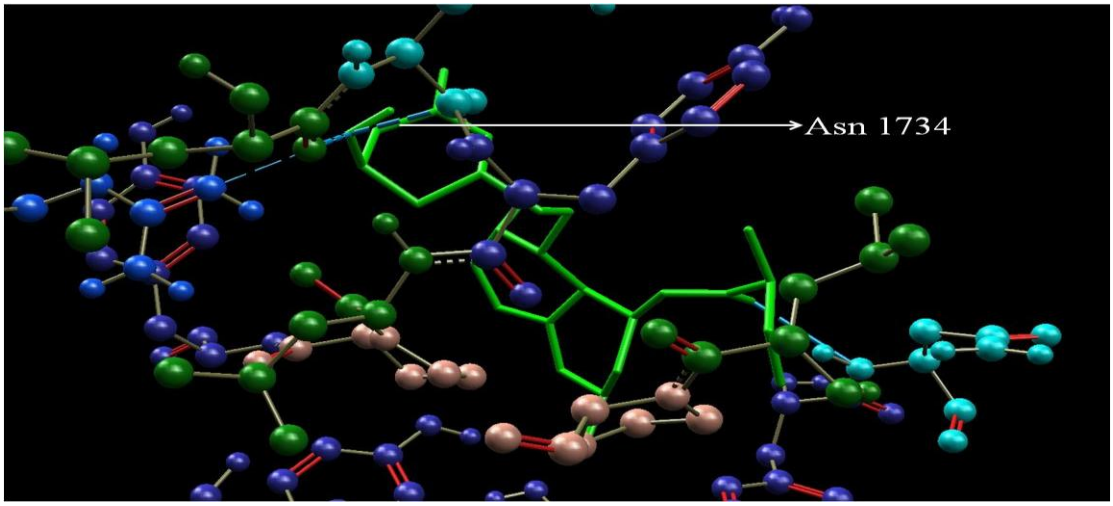

Figure 2. Binding of proteins exhibiting the inhibition of HMG Co A reductase.

lovastatin on inhibition of HMG-Co A reductase enzyme (Figure 2) in order to reduce the blood cholesterol level. Docking studies showed that lovastatin was bound to the active region of reductase enzyme (PDB ID: 1DQ9) (Istvan et al., 2000), thereby inhibiting them. A hydrogen bond interaction was made with Asn amino acid residue at 1734 position with the oxygen atom in the ligand, the length between the oxygen and Asn at 1734 position is 3.0633 and the energy utilized for the interaction is -2.5kcal/mol. Another hydrogen bond interaction was made in ligand with the protein; the interaction was between the oxygen in the lactone ring and to the Leu and Arg amino acid residue at 1319 and 1306 positions, respectively in the protein. The energy utilized for the interaction of ligand with Leu 1319 (-2.4273 kcal/mol) is much higher than with Arg 1306 (-0.7036 kcal/mol). Thus, three interactions occur between lovastatin and the protein, where the interaction between Asn and Leu were more effective. The overall binding energy between the ligand and the HMG Co A is -11.2kcal/mol. This proves that the interaction occurs between lovastatin and HMG Co A reductase, was more effective and helps in the reduction of cholesterol levels in blood by inhibiting the HMG Co A reductase enzyme (PDB ID: 1DQ9).

Alzheimer's disease

Interaction between lovastatin and Tau protein (PDB ID: 1J1B)

Tau proteins are the macromolecules which play a vital role in regulation of microtubules in brain and central nervous system (CNS). Malfunction of Tau protein leads to dementia such as Alzheimer's disease. This can be regulated by binding various drugs with Tau protein (PDB ID: 1J1B). Lovastatin was docked with Tau protein (Figure 3), to know the effectiveness of the drug to cure Alzheimer disease. Docking of lovastatin with Tau protein showed (Figure 3) a hydrophilic interaction at the lactone ring of lovastatin with Pro 636, Arg 641 and Tyr 634; the binding energy was -2.5, -2.0 and -2.5 kcal/mol, respectively, and the length of the hydrogen bond between ligand and Pro 636, Arg 641, Tyr 634 are 2.77, 3.19, 3.05, respectively. From this, it is clear that the chance of interaction will be high with Tyr 634 position than with Arg and Tyr at position 641 and 634. Another interaction occurs at the free oxygen end of the ligand with Tau protein at 564 position of Asn amino acid, the binding energy is -0.29 kcal/mol and the length between them is 3.416. Thus the interaction is effective with Tyr at position 634 and Asn at 564 position, which shows that lovastatin binds with Tau protein, but its effectiveness of the interaction is not well known since the inhibition may affect the formation of microtubules and its stabilization.

Interaction between lovastatin and TNF α protein (PDB ID: 2AZ5)

TNF-α protein belongs to cytokine group, where it plays a role in systemic regulation of inflammation. The dysfunction of TNF-α may leads to various disease like Alzheimer's, cancer, inflammation bowel disorders. Many drugs were designed to target particular sites of TNF-α protein at the particular sites for the regulation of the protein and to reduce the diseases. Docking was carried out with lovastatin and TNF-α protein (PDB ID: 2AZ5) are shown in Figure 4, to treat Alzheimer's disease. Docking results showed two hydrogen bond interaction with lovastatin, where a single interaction occurs with the oxygen atom present in lactone ring of lovastatin to Tyr 552 and with the Gly 382 aminoacid residues of TNF α protein, the binding energy between Tyr and ligand at

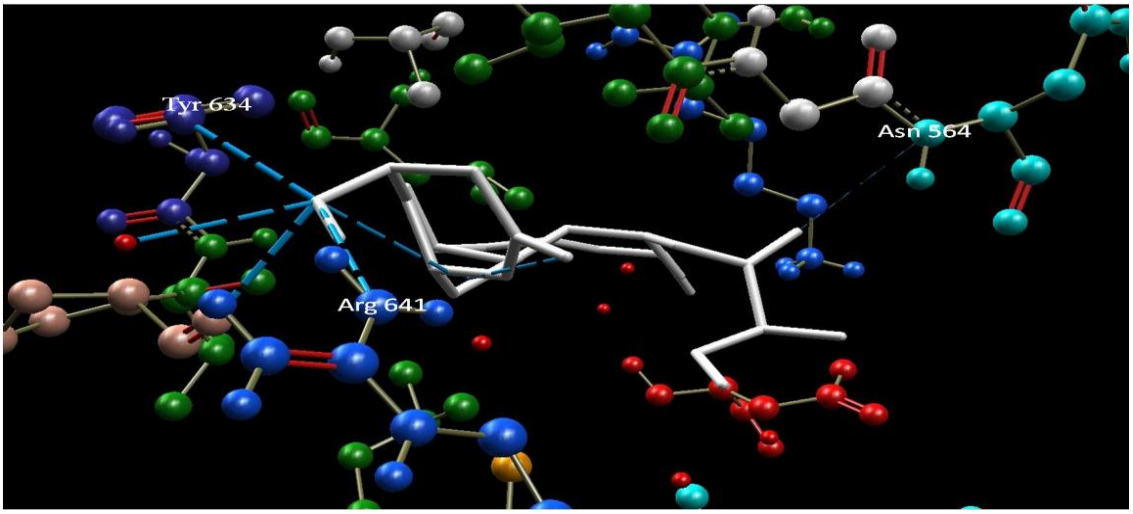

Figure 3. Binding of Lovastatin and Tau protein.

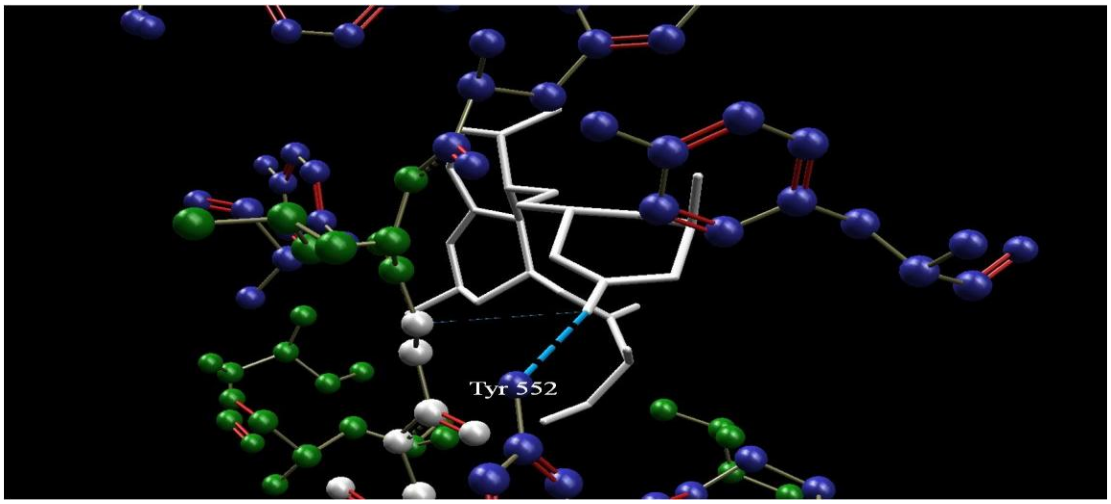

Figure 4. Binding of Lovastatin and TNF α**.**

position 552 is -2.5kcal/mol and the length between them is 2.70. Another interaction occurs at the same position of the ligand molecule with Gly at position 382 and the hydrogen bond interaction is -0.2 kcal/mol. Thus the interaction will be maximum only with Tyr molecule than with Gly since the energy used and the interaction between them is stronger. The overall energy utilized for the hydrogen bond interaction is -7.9 kcal/mol, which is slightly higher than the interaction with Tau protein. Thus lovastatin may reduce Alzheimer's disease in humans by inhibiting TNF α protein at the specific sites.

Interaction between Lovastatin and TNF β protein (PDB ID: 1TNR)

TNF-β is a similar kind of protein as TNF-α, where it shows 30% homologues to one another. The interaction of lovastatin with TNF-β protein (1TNR) was not that efficient (Figure 5), only one hydrogen bond interaction took place at the lactone ring position with the oxygen molecule and also the energy utilized for the overall interaction is -7.6kcal/mol, which is much higher compared with the interaction of other proteins with lovastatin. The hydrogen bond interaction was weak and the chance of interaction is very much limited than compared with the other proteins such as Tau, TNF-α.

Effect of lovastatin on Alzheimer's disease

Docking studies were done with various proteins responsible for Alzheimer's disease with lovastain. In this, a maximum interaction was made with Tau protein (1J1B)

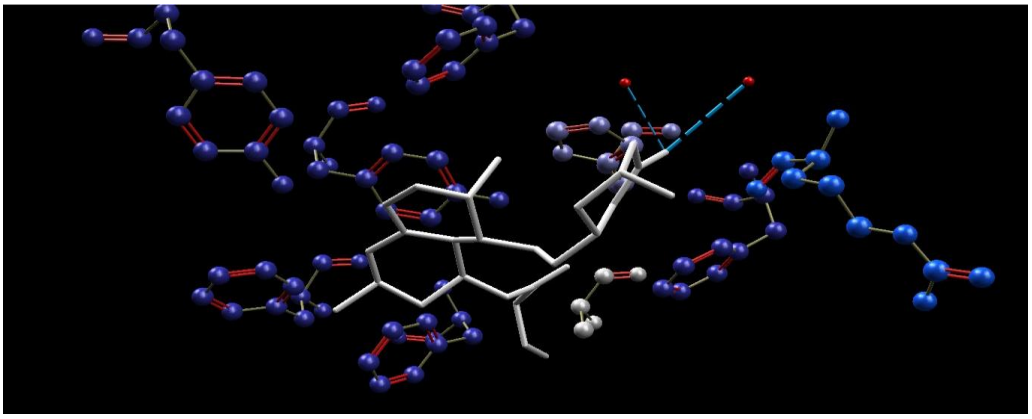

Figure 5. Binding of lovastatin and TNF β.

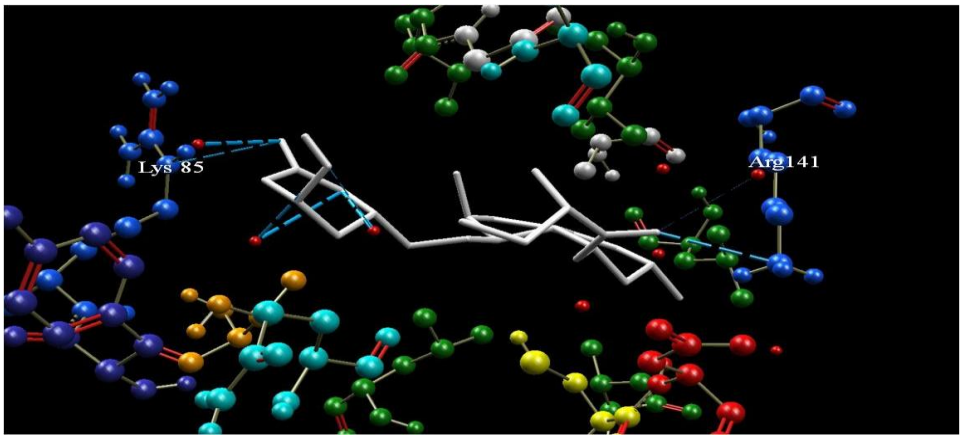

Figure 6. Binding of lovastatin and glycogen synthase kinase-3β.

than with other two proteins, but the interaction occurs at the same region in the lovastatin molecule. TNF-α had only two interactions with lovastatin and the energy used was also minimum, the interactions was very effective than with other two proteins. So the drug may interact more effectively with TNF- α protein than with Tau, TNF-β proteins. Thus the drug may help in curing Alzheimer's disease by interaction with TNF-α protein with the specific amino acids at particular sites.

Cancer proteins

Interaction between lovastatin and glycogen synthase kinase-3β (PDB id: 1UV5)

Glycogen synthase kinase-3β (GSK -3β) (Meiger et al, 2003) mediates the addition of phosphate molecules to serine and threonine residues. GSK -3β phosphorylates at the active sites of serine, threonine residues. The active sites of GSK- 3 β are present at 181, 200, 97 and 85 positions. GSK-3β has a role in apoptosis and also studies showed that it has a role in cancer formation (Luo, 2009.). Certain cancer can be treated by inhibition of GSK-3β, lovastatin has been shown to inhibit GSK-3β at active sites (Figure 6). The docking studies showed that lovastatin binds to GSK-3β at different sites with a binding affinity of -8.1 kcal/mol. Lovastatin binds to Lys residue at 85 position, which is one of the active site present in GSK-3β. The hydrogen bond interaction utilizes the energy of -2.10kcal/mol and the bond length of 3.17 at 85th position. Another interaction occurs at the oxygen of the lactone ring with Arg aminoacid residue at 141th position. The site where it binds is not an active site, so the interaction may not have any influence over the inhibition of GSK- 3β. Thus lovastatin may act as an anti-cancer drug by inhibiting GSK-3β at position 85 and 141, where the interactions occur more effectively and also, they are the active sites present in GSK- 3β proteins

Interaction between lovastatin and thymidylate synthase (PDB id: 1JU6)

Thymidylate synthase is a key in formation of thymidine

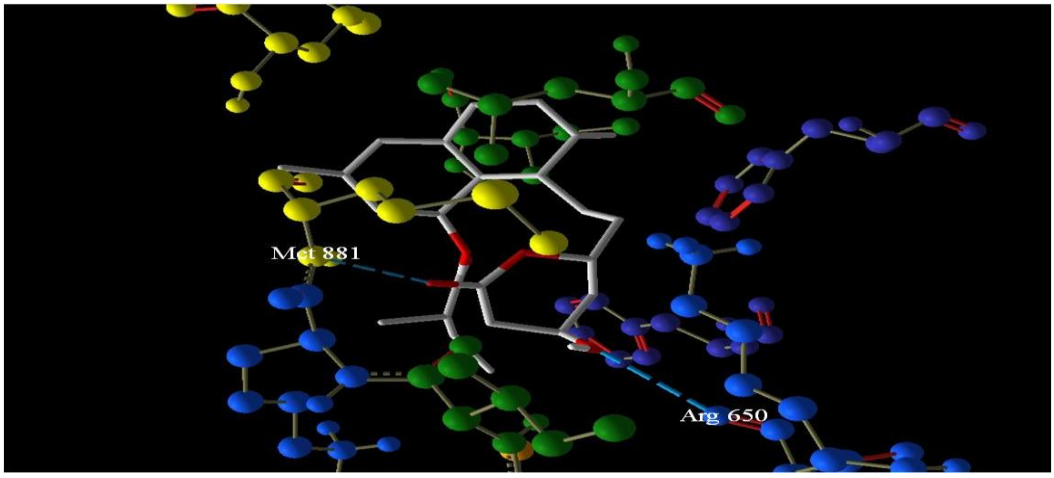

Figure 7. Binding of lovastatin and thymidylate synthase.

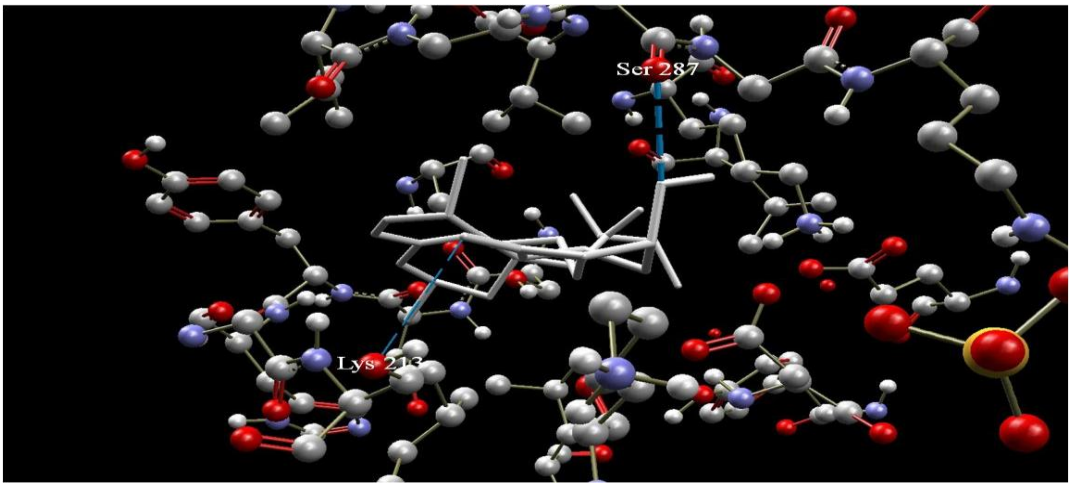

Figure 8. Binding of lovastatin and TGF-β receptor type I.

monophosphatae, which in turn is used in the synthesis and repair of DNA (Sayre et al., 2001). They also play a significant role in liver cancer proliferation, where thymidylate synthase was induced by Late SV40 (LSF). The inhibiton of this protein may stop the cancer induction in liver cells of humans. Docking study of lovastatin with thymidylate synthase showed an interaction between them (Figure 7).

Lovastatin binds thymidylate synthase at 881 and 650 positions with Met and Arg residues. Where a hydrogen bond interaction takes place with the oxygen atoms in the lactone rings, the hydrogen bond interaction between the aminoacid and ligand used a energy of -7.7 kcal/mol. The energy utilized was high since the binding regions are not an active site and also the hydrogen interaction was a weaker one at that particular site. From the docking studies, it is clear that the chance of interaction between lovastatin and thymidylate synthase is weak. This study proves that the ligand may not bind with thymidylate synthase for the treatment of cancer in human.

Interaction between lovastatin and TGF-β receptor type I (PDB id: 1PY5)

TGF-β receptor (PDB id: 1PY5) is found in the tissue types including brain, kidney, liver and testes. They also play a key role in proliferation and differentiation of many cell types. A change in protein may induce cancer in human cell. By inhibition of these cells, it may help in the regulation of cancer in human cells. Lovastatin was docked with TGF-β receptor protein (Figure 8), to find its effectiveness in inhibition for the treatment of cancer.

A binding occurs in protein at position 213 and 287, with lovastatin ligand on the lactone ring sites. The hydrogen interaction is higher and also elctrostatic interactions were found between them. The hydrogen bond formation between the ligand and the protein used energy of -9.5 kcal/mol, where the ligand binds to Lys and Ser residues of the protein. The interaction was stronger between them and so the inhibition of TGF-β receptor may occur effectively by binding of lovastatin at

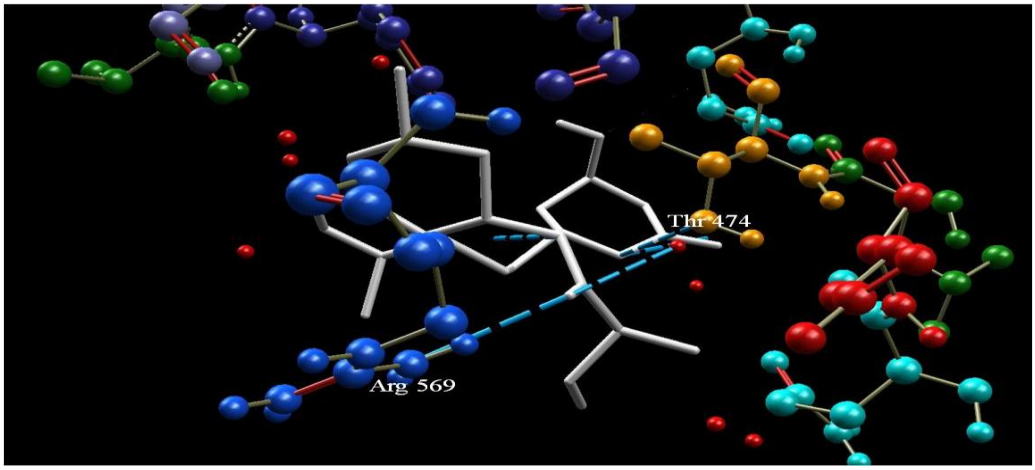

Figure 9. Binding of lovastatin and focal adhesion kinase 1.

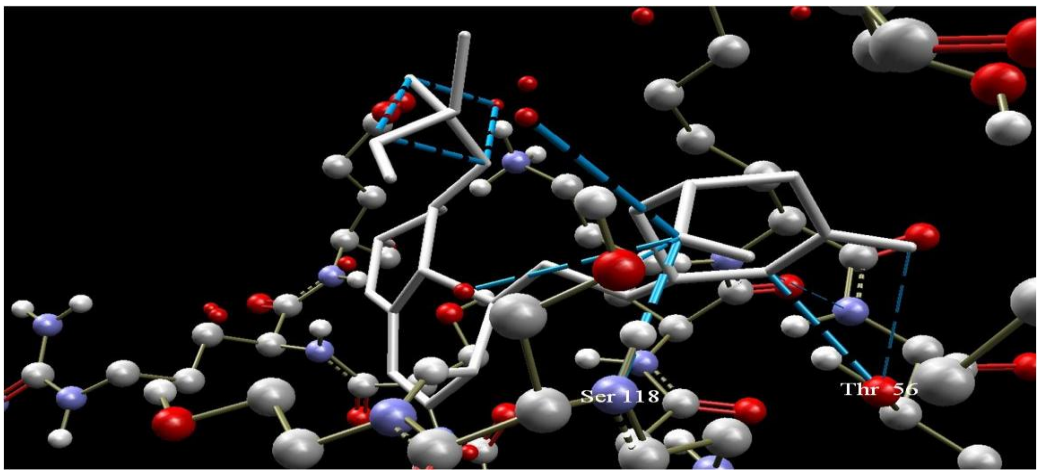

Figure 10. Binding of lovastatin and dihydrofolate reductase.

the targeted sites and thereby reducing the cancer effects in human.

Interaction between lovastatin and focal adhesion kinase 1 (PDB id: 3BZ3)

Focal adhesion kinase 1 (FDK-1) (PDB id: 3BZ3) is a non receptor protein that plays a vital role in cell adhesion, migration and cell proliferation. They also play a very vital role in cell apoptosis. An increase in FDK-1 may lead to cancer in the cells, which may be due to cell adhesion and proliferation nature of FDK-1. By inhibiting FDK-1 with lovastatin, it may regulate cancer in human. Lovastatin showed a mild interaction with FDK-1 protein (Figure 9), when interacted at 464 and 569 positions. The energy utilized for the hydrogen bond interaction at Arg and Thr residues is -7.1 kcal/mol. More energy was utilized during the hydrogen bond interaction, which may be due to the other interaction which occurred with the hydrogen molecules present in the FDK-1 protein. Further studies are required to study the effectiveness of lovastatin with FDK-1 proteins, for future use as an anti-cancer drug.

Interaction between lovastatin and dihydrofolate reductase (PDB id: 3GI2)

Dihydrofolate reductase (PDB id: 3GI2) is an enzyme that helps in the reduction of tetrahydrofolic acid to dihydro folic acid. Many drugs are designed targeting dihydrofolate reductase enzyme, since it plays a role in proliferation of cancer cells. Docking studies showed that lovastatin also targets dihydrofolate reductase enzyme (Figure 10) at a specific site with an energy utilization of -6.7. Lovastatin binds at 118 and 56 positions, with Ser and Thr residues, at hydrogen bond length of 3.34 and

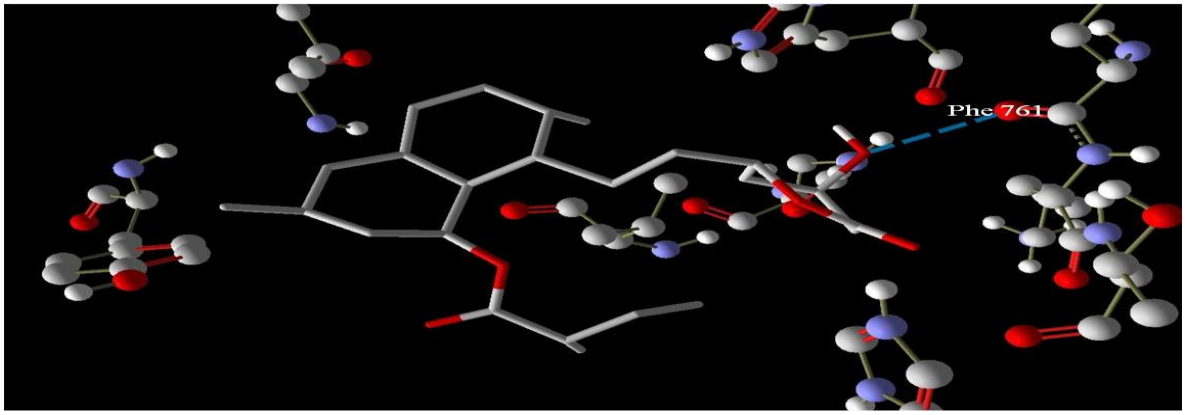

Figure 11. Binding of lovastatin and cyclooxygenase.

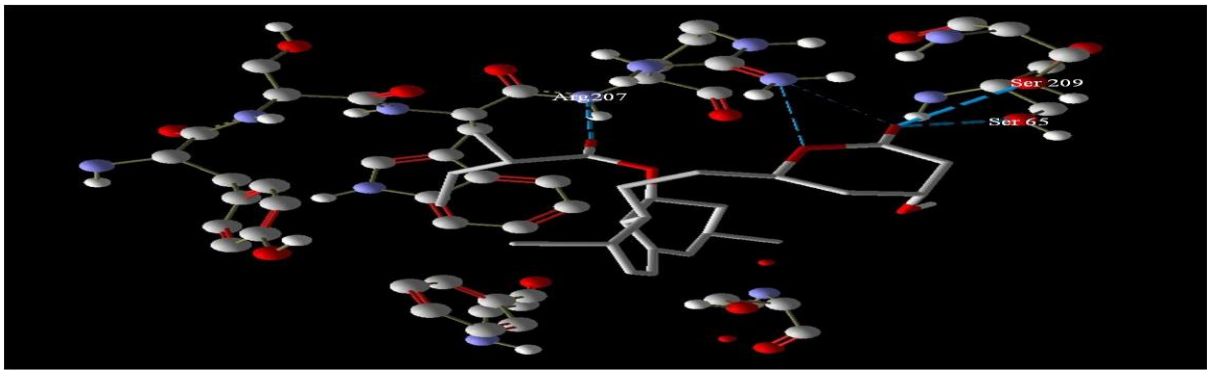

Figure 12. Binding of lovastatin and caspase-3.

2.87. Energy utilized for the bond formation is -1.26 and -2.5 kcal/mol, respectively. The binding of lovastatin with protein utilizes more energy and the bond may have an interaction with other hydrogen molecules present in the enzymes. Thus lovastatin may inhibit the cancer protein by inhibiting dihydrofolate reductase enzyme, by which lovastatin can be used in the treatment of cancer.

Inhibition of cyclooxygenase using lovastatin (PDB: 1CX2)

Cyclooxygenase (COX) is an important enzyme in formation of prostaglandins. COX plays a vital role in inflammation and so many drugs were designed to inhibit the enzyme. In our study, lovastatin's anti-inflammatory property was checked using Autodock Vina with COX (PDB: 1CX2). The docking showed a binding of lovastatin with COX protein at a single site (Figure 11), 761 positions with Phe residue. A single hydrogen bond interaction with the protein was not efficient when docking was made with other proteins. The study shows that the lovastatin may not be much efficient for the treatment of anti-inflammatory drugs.

Apoptosis

Docking of lovastatin with Caspase-3 (PDB ID: 1GFW)

Caspase is an essential protein in apoptosis. Dysfunction of caspase protein may lead to tumor and inhibition of caspase-3 (PDB ID: 1GFW) which helps in treating tumor in humans (Lee et al., 2000). In our study, lovastatin was docked with caspase protein to check its efficiency in treatment of tumor in humans (Figure 12). The study reveals that lovastatin binds at 65, 209 and 207 positions, with Ser and Arg residues. The binding energy utilized was -7.7 kcal/mol, which was very high for the binding of lovastatin with caspase enzyme. Thus the studies reveal that, lovastatin binds with caspase protein but not efficient enough for the treatment of cancer in humans, by interacting with caspase-3.

Docking of lovastatin with cyclin-dependent protein kinase-7 (PDB ID: 1UA2)

Cyclin-dependent protein kinase-7 (CDK-7) binds with lovastatin at two sites, 257 and 258 positions, with Leu and His residues (Figure 13). The energy utilized for

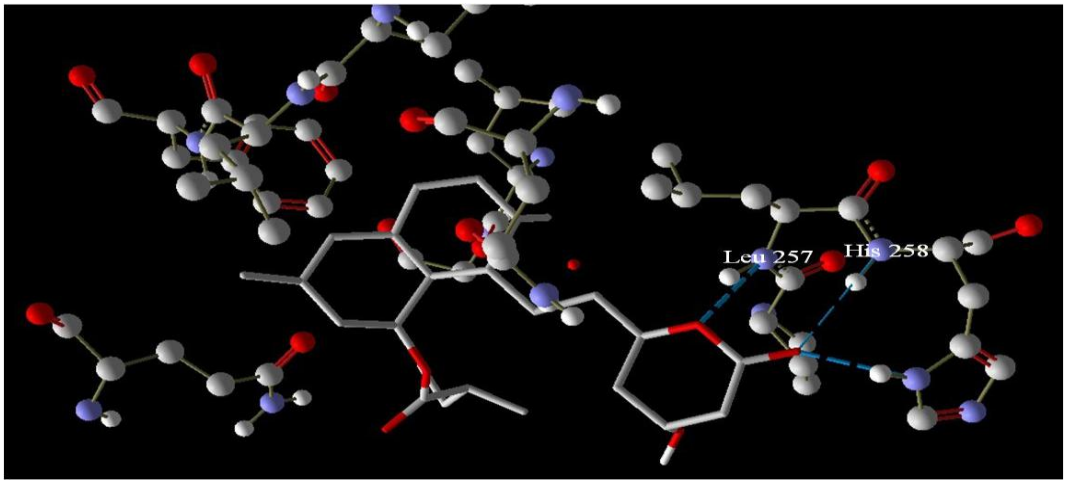

Figure 13. Binding of lovastatin and cyclin-dependent protein kinase-7.

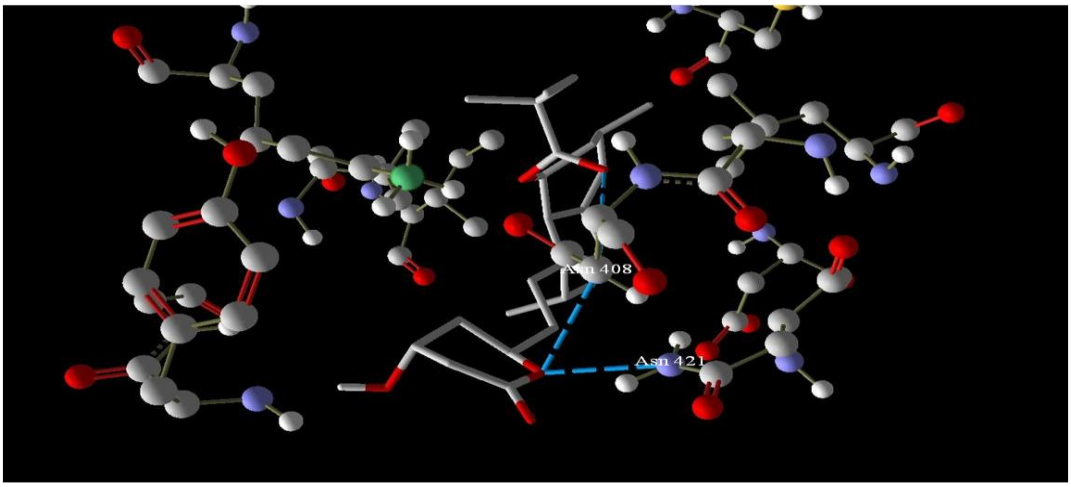

Figure 14. Binding of lovastatin and cyclin-dependent protein kinase-5.

hydrogen bond interaction is -7.6 kcal/mol, where the bond length is 2.83 and 2.57 and the energy used for each interaction is -2.5 and -2.1 kcal/mol, with Leu and His residues, respectively. The hydrogen bond interaction of lovastatin was not efficient enough to inhibit CDK-7 and also the energy utilized is much higher for the interactions to occur. Thus the mechanism of inhibiting CDK-7 is not an efficient method for the treatment of cancer using lovastatin.

Cyclin-dependent protein kinase-5 (CDK-5) is a similar kind of protein as CDK-7, which has similar properties in regulation of cells. The CDK-5 was docked with lovastatin molecules (Figure 14), where it binds to Asn amino acid residues at position 408 and 421.The energy utilized for the hydrogen bond interaction is -8.2 kcal/mol, where the energy utilized for binding at the active site is more when compared with docking studies obtained from other cancer proteins. The results show that the chances of lovastatin acting over CDK-5 is much less compared with other proteins for the treatment of cancer cell.

Docking of lovastatin with cyclin-dependent protein kinase-2 (PDB ID: 2UZO)

Cyclin-dependent protein kinase-2 (CDK-2) also plays a vital role in regulation of cell cycle, apoptosis. Docking of lovastatin showed two hydrogen bond interactions with CDK-2 at 42 and 122 positions with His and Gly residues (Figure 15). The hydrogen bond interaction utilized a binding energy of -8.2 kcal/mol, which is very high for the interaction with apoptosis proteins. Thus the inhibition of CDK-2 will also be less efficient for the interaction with lovastatin drug. Since the energy utilized is much higher

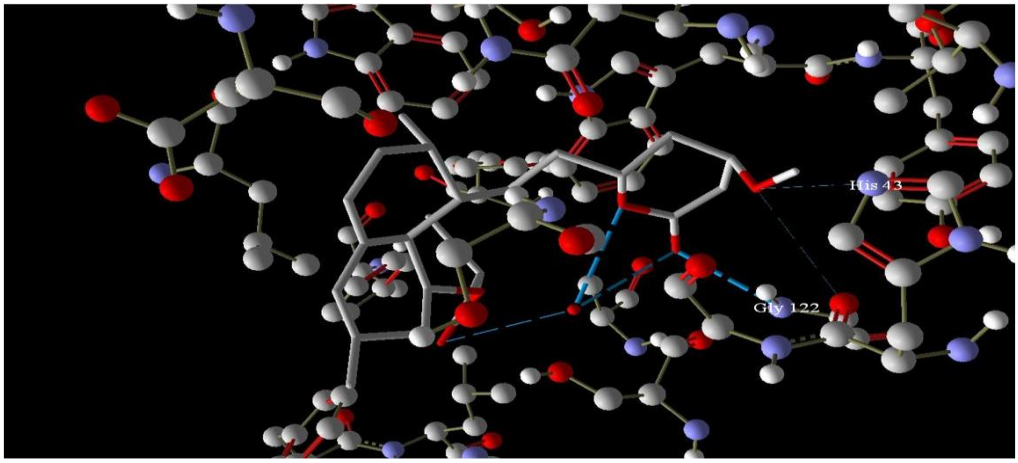

Figure 15. Binding of lovastatin and cyclin-dependent protein kinase-2.

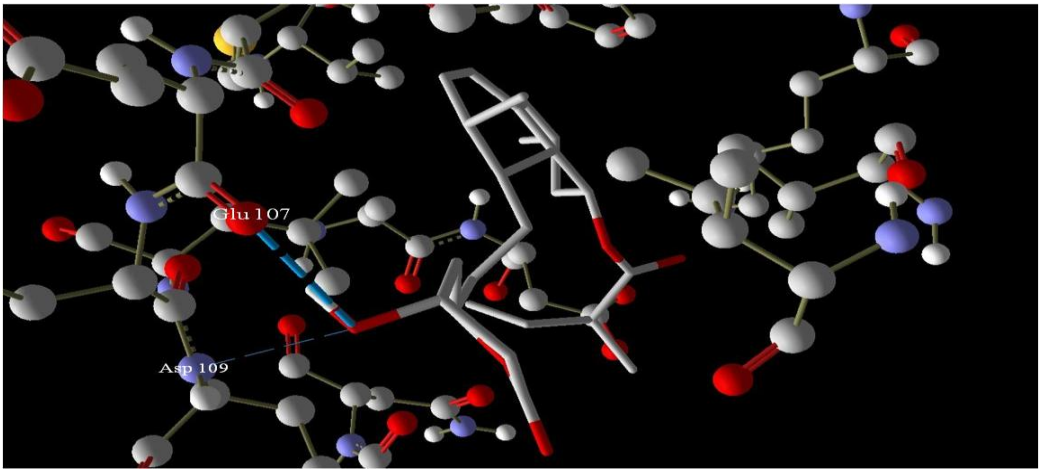

Figure 16. Binding of lovastatin and cyclin-dependent protein kinase-9.

for the interaction, the drug may bind with protein molecules other than CDK-2 proteins.

Docking of lovastatin with cyclin-dependent protein kinase-9 (PDB ID: 3BLR)

Docking of lovastatin with cyclin-dependent protein kinase-9 (CDK-9) was carried out, the results obtained showed the binding sites of lovastatin with CDK-9 at 107 and 109 positions. The hydrogen binding interaction utilized -8.7 kcal/mol of energy, where the binding occurred only at one site in the lovastatin drug as shown in Figure 16. This interaction was not that efficient since two hydrogen bond interaction occurs at same place in the lovastatin drug. Thus lovastatin's mechanism of action over cancer may not be induced by inhibiting CDK-9 proteins, since this interaction consumes more energy and also the hydrogen bond interaction was not that efficient in inhibition of CDK-9 proteins.

Conclusion

The docking studies reveal that lovastatin has various properties like anti-lipidemic, anti-cancer and Alzheimer's disease. The mechanism of lovastatin drug as anti-lipidemic, occurs by inhibiting HMG-Co A enzyme, which is a precursor in cholesterol synthesis. Similarly, lovastatin can help in curing alzheimer's disease, by inhibiting the Tau protein than other proteins like TNF-α and TNF-β. The docking study was extended to check the anti-cancer property of lovastatin, where ten proteins were docked and results showed that the inhibition of cancer cell by lovastatin was efficient by inhibiting TGF β receptor protein.

ABBREVIATIONS

PDA, Potato dextrose agar; **TNF,** tumor necrosis factor; **CNS,** central nervous system; **GSK -3β,** glycogen synthase kinase-3β; **COX,** cyclooxygenase.

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Full Length Research Paper

Bioactivity of gentamicin contained in novel transdermal drug delivery systems (TDDS) formulated with biodegradable polyesters

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Topical administration of gentamicin, a hydrophilic aminoglycoside antibiotic, is limited by membrane impermeability and toxicity concerns. The purpose of this study was to develop and evaluate the antimicrobial activities of an alternative non-invasive, convenient and cost-effective transdermal drug delivery system (TDDS) containing gentamicin in biodegradable polyester-based matrices. The patches were formulated by solvent evaporation technique using PURASORB® polymers and evaluated for thermal properties, drug content, physicochemical performance, stability, skin irritation on rat skin and antimicrobial activities against five micro-organisms: *Staphylococcus aureus, Escherichia coli, Salmonella typhi*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae*. The differential scanning calorimetry (DSC) results indicated compatibilty between the drug and the polymers. In addition, the formulations showed good drug encapsulation, stability, physicochemical properties, tolerability on rabbit skin and higher zones of inhibition compared with a commercially available gentamicin sulphate cream against *S. aureus, E. coli, S. typhi* and *P. aeruginosa*, while *K. pneumoniae* was mildly susceptible. Compared with the rest of the formulations, patches of PURASORB® PL 32 exhibited the best stability, tolerability on rat skin and bioactivity. This study has shown that transdermal patches of PURASORB® PL 32 represent an alternative delivery system for gentamicin for treatment of infections caused by gentamicin-susceptible micro-organisms.

Key words: Antimicrobial activities, bioadhesive strength, gentamicin, PURASORB® polymers, transdermal patches.

INTRODUCTION

Transdermal drug delivery system is being extensively investigated as a viable alternative to drug delivery with improved bioavailability. Transdermal drug administration generally refers to topical application of agents to healthy

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intact skin either for localized treatment of tissues underlying the skin or for systemic therapy (Valenta and Auner, 2004). It offers many advantages over conventional administration such as enhanced efficacy, increased safety, and greater convenience and improved patient compliance (Valenta and Auner, 2004; Dnyanesh and Vavia, 2003; Chandak and Verma, 2008). Transdermal route permits the use of a relatively potent drug with minimal risk of system toxicity and avoids gastrointestinal degradation and hepatic first-pass metabolism (Mundargi et al., 2007; Mutalik and Udupa, 2004). In case of toxicity, the transdermal patch can easily be removed by the patient (Chang et al., 2006).

Gentamicin sulphate is an aminoglycoside antibiotic commonly used topically in the control of severe Gram positive and Gram negative microbial infections especially in burns and wounds as well as for treating bone and soft tissue infections (Nishijima and Kurokawa, 2002). Despite its benefits, bacterial barriers and adverse effects such as nephrotoxicity, ototoxicity and neurotoxicity upon prolonged use, limit gentamicin daily dosage (Drusano et al., 2007). In fact, many clinicians are reluctant to use it, even for a short term (Singh et al., 2003). Efforts have been made to determine its optimal therapeutic regimens in order to increase its overall efficacy while minimizing drug toxicity. These include various formulation techniques such as formulations for topical administration.

Though topical administration of gentamicin is not painful, the patient may not apply the ointment/cream as often as required. Therefore, there is a need for the preparation of a new dosage form of gentamicin. Owing to the advantages offered by transdermal drug delivery systems (TDDS) over conventional routes of administration (Mundargi et al., 2007; Mutalik and Udupa, 2004), this study was designed to evaluate the transdermal delivery system of gentamicin so as to develop non-parenteral and needle-less (non-invasive) gentamicin preparation that will not only reduce to the barest minimum the systemic toxicity associated with parenteral administration of gentamicin but also ensure patient's compliance. A transdermal patch is not painful and is cost effective (Chang et al., 2006). Technically, the patch is placed in a part of the body which releases the drug into the body for a long period of time (Valenta and Auner, 2004; Dnyanesh and Vavia, 2003; Chandak and Verma, 2008). Polymeric matrices are usually employed as carriers for transdermal delivery of drugs/actives (Dnyanesh and Vavia, 2003; Chandak and Verma, 2008; Mundargi et al., 2007; Mutalik and Udupa, 2004; Verma and Iyker, 2002; Gupta and Mukherjee, 2003; Lyman, 2007). The novelty embodied in this study lies in the formulation of gentamicin transdermal patches using PURASORB® polymers, a well-established, safe, biocompatible and resorbable excipients commonly employed in the formulation of controlled release drug delivery systems. These biodegradable polyesters have wide applications, including as orthopedic implant devices, surgical sutures, cardiovascular products, tissue regeneration scaffolds, among others. PURASORB® materials allow for maximum flexibility in formulation technologies, ranging from extrusion and solvent processing to spray drying. Moreover, they are the material of choice for the production of implants, microspheres, and depot systems (Yasukawa et al., 2001; Avitable et al., 2001; Arora and Mukherjee, 2002).

The objective of this study, therefore, was to design and formulate transdermal patches incorporating gentamicin using biodegradable polyesters for the purpose of enhancing the delivery of the drug, by providing controlled delivery of the drug. The suitability of four different biodegradable polyesters (PURASORB® polymers: PLGA, PDL 05, PL 32 and PDL 04) for this purpose was assessed by evaluating some of the physicochemical properties of the patches formed, the efficiency of incorporation of the drug in the patches as well as the bioactivity of the incorporated drug.

MATERIALS AND METHODS

The following materials were used without further purification: Gentamicin (Schering, Rockville, MD, USA), Poly(D, L-lactide-co-glycolide PLGA)-PURASORB® PDLG 7502A, poly(L-lactide) -PURASORB PL® 32, poly(DL-lactide)-PURASORB® PDL 04, and poly(DL-lactide) -PURASORB® PDL 05 (PURAC biochem by Gorinchem, Holland), ethyl acetate, sodium borate, ophthalidialdehyde and 2-mercaptoethanol (Sigma-Aldrich, USA), polyvinyl alcohol, propylene glycol (Merck, Germany), isopropanol, methanol and formalin (Adwic El-Nasr, Chemical Co., Cairo, Egypt), sodium hydroxide (BDH, England) and distilled water (Lion water, UNN, Nigeria). Clinical isolates of *Staphylococcus aureus* ATCC 13703, *Salmonella typhi* ATCC 786 and *Escherichia coli* ATCC 9637 were obtained from Bishop Shanahan Hospital, Nsukka, Nigeria; whereas laboratory isolates of *Pseudomonas aeruginosa and Klebsiella pneumoniae* were procured from the Microbiology Laboratory, Department of Pharmaceutics of our University. Gentamicin sulphate cream USP, 0.1% (Perrigo Bronx, New York, USA) was used as a commercially available topical gentamicin cream. All other laboratory materials were of analytical grade. All experiments involving the use of animals were conducted in accordance with Ethical Guidelines of Animal Care and Use Committee (Research Ethics Committee) of University of Nigeria, Nsukka, following the 18th WMA General Assembly Helsinki, June 1964 and updated by the 59th WMA General Assembly, Seoul, October 2008.

Preparation of transdermal patches

The patches were prepared by solvent evaporation technique (Amunuaikit et al., 2005), using gentamicin, plasticizers and other film forming polymers. Gentamicin (5 g) was dissolved with distilled water (5 ml) in a beaker followed by addition of 10 g of propylene glycol. The mixture was stirred continuously until a solution (drug reservior) was formed. The backing membrane was cast by weighing approximately 10 g of the film forming polymer (PLGA) into a separate beaker, adding about 70 ml of ethyl acetate and

Table 1. Composition of transdermal patches.

Ingredient	Formulation code			
	PLGA	PDL 05	PL 32	PDL 04
Drug (g)	5	5	5	5
Polymer (g)	10	10	10	10
Propylene glycol (g)	10	10	10	10
Water (ml)	5	5	5	5
Chloroform (ml)	...	...	180	...
Ethyl acetate (ml)	70	63	...	180
0.1N NaOH (ml)	...	...	5	5

votexing (Vortex Genie Bouxemia. N.Y 11716, USA) the mixture for 5 min. Subsequently, the drug-contaning solution (drug reservior) was then poured into the solution containing the PLGA (backing membrane). This dispersion was properly stirred and poured into an aluminium foil-lined petri dish of defined area (10 cm^2). A funnel of suitable size was inverted over the petri dish to minimize solvent evaporation. Casting solvent was then allowed to evaporate 48 h to obtain dry films. The above procedure was repeated using PDL 05, PL 32 and PDL 04 as the film forming polymers as well as appropriate quantities of either or both ethylacetate, chloroform and sodium hydroxide (0.1N NaOH), as depicted in Table 1. The patches were stored between sheets of wax paper in a desiccator until further analysis.

Differential scanning calorimetry (DSC)

The stability and compatibility of gentamicin and different polymers to be used for the development of gentamicin transdermal film formulations was studied using a differential scanning calorimeter (Netzsch DSC 204 F1, Germany). Sample (2.5 – 5 mg), placed in an aluminum crucible cell was firmly crimped with the lid to provide an adequate seal. The thermal properties such as melting temperature, enthalpy and glass transition of the drug and transdermal patches were deteremined in the range of 35 – 190°C under a 20 ml/min nitrogen flux at a heating rate of 10°C/min. The baselines were determined using an empty pan, and all the thermograms were baseline corrected.

Characterization of gentamicin transdermal films

The patches were characterized according to standard procedures with respect to physical appearance, thickness (Devi et al., 2003), weight variation (Gupta and Mukherjee, 2003), moisture content (Chang et al, 2006), moisture uptake (Lyman, 2007), film folding endurance (Zhang et al., 1994), drug content (Chang et al., 2006; Bazigha et al., 2011), *in vitro* bioadhesive strength (Ganesh et al., 2011; Verma and Chandak, 2009) and skin irritancy (National Committe for Clinical Laboratory Standards, 2003).

Bioevaluation of transdemal patches

The antimicrobial activity of the transdermal patches was tested against each isolates using the agar diffusion technique (Umeyor et al., 2011). This method depends on the diffusion of antibiotics from holes on the surface of the microbial seeded agar. This test was carried out for the prepared gentamicin patches as well as the commercially available gentamicin sulphate cream. Molten nutrient agar (20 ml) was inoculated with 0.1 ml of *S. aureus* broth culture. It was mixed thoroughly, poured into sterile Petri dishes and rotated for even distribution of the organism. The agar plates were allowed to set and a sterile cork borer (8 mm diameter) was used to bore holes in the seeded agar medium. For the tested patch formulae as well as the commercially available cream, a definite quantity containing equivalent amount of gentamicin was accurately weighed and inserted in a corresponding hole. The plates were allowed to stand at room temperature for 15 min to enable prediffusion before incubating at 37 ± 0.5°C for 24 h. The experiment was repeated for *E. coli*, *P. aeruginosa, S. typhi* and *K. pneumoniae*. Three replicate tests were performed in each case. Growth was examined after incubation and the diameter of each inhibition zone was measured and the average determined.

Stability study of transdermal patches

Time resolved stability studies were carried out on the various batches of the formulations following the ICH guidelines (Verma and Chandak, 2009). The transdermal patches were stored at 40 ± 0.5°C in a humidity chamber having a RH of 75 ± 5%. After four weeks, six months and one year of storage, the patches were withdrawn and evaluated for the drug content following the method stated above.

Statistical analysis

All experiments were performed in replicates for validity of statistical analysis. Results were expressed as mean ± SD. ANOVA and Student's t-test were performed on the data sets generated using Statistical Package for the Social Sciences (SPSS). Differences were considered significant for p-values < 0.05.

RESULTS AND DISCUSSION

Differential scanning calorimetry (DSC)

Table 2 presents the thermal properties of the transdermal patches. The physicochemical compatibility of the drug and the polymers studied by differential scanning calorimetry suggested absence of any incompatibility. The results revealed the compatibility of gentamicin and the polymers as well as the stability of the drug in the polymeric matrices (formulations). This is because the formulations gave lower endotherms than gentamicin,

Table 2. Thermal properties of gentamicin transdermal patches.

Formulation code	Thermal properties				
	Melting point (ºC)	Enthalpy (Mw/mg)	Glass transition temperature (T_g) (ºC)		
			Onset	Middle	End
PLGA	-	-	31.3	26.9	22.4
PDL 05	-	-	38.7	40.1	41.5
PL 32	180.7	-1.981	80.2	89.0	97.8
PDL 04	126.5	-5.155	39.9	42.9	46.0

PL 32, PDL 04, PDL 05 and PLGA are gentamicin-loaded patches containing PURASORB® PL 32, PDL 04, PDL 05 and PLGA, respectively.

Table 3. Properties of gentamicin transdermal patches

Parameter	Formulation code			
	PLGA[a,b]	PDL 05[a,b]	PL 32[a,b]	PDL 04[a,b]
Thickness (µm)	390.50 ± 9.08	400.60 ± 2.18	384.20 ± 5.97	405.80 ± 6.03
Weight variation (mg)	20.90 ± 0.27	21.30 ± 0.25	20.70 ± 0.01	21.60 ± 0.15
Drug content (%)	97.60 ± 2.43	95.80 ± 1.49	98.90 ± 2.45	96.80 ± 2.42
Folding endurance	316.50 ± 4.17	327.10 ± 3.89	307.90 ± 5.02	311.40 ± 8.09
Tensile strength (dyne cm^{-2})	75.60 ± 1.07	68.70 ± 1.04	81.40 ± 2.03	60.50 ± 1.40
Moisture absorption (%)	2.70 ± 0.28	2.90 ± 0.67	1.90 ± 0.33	2.60 ± 0.20
Moisture content (%)	1.60 ± 0.29	1.20 ± 0.07	1.50 ± 0.14	1.70 ± 0.38

[a]Mean±SD, [b]n=3, PL 32, PDL 04, PDL 05 and PLGA are gentamicin-loaded patches containing PURASORB® PL 32, PDL 04, PDL 05 and PLGA, respectively.

implying that gentamicin exists in amorphous state in the formulations and also is properly solubilized in the matrix systems (Nnamani et al., 2010). Moreover, there was general disappearance of drug peak in all patches but PURASORB® PL 32 patch had the least enthalpy (-1.981 mW/mg) reflecting the degree of disorder in the matrix.

Characterization of gentamicin transdermal films

The results of the physicochemical characterization of the patches are shown in Table 3. All the patches of the different polymers were transparent, colourless, smooth and uniform but the PDL 05 polymer had the greatest clarity. The results indicated that the formulated gentamicin-loaded patches exhibited good organoleptic and physicochemical properties (Valenta and Auner, 2004; Dnynesh and Vavia, 2003; Chandak and Verma, 2008; Mundargi et al., 2007; Mutalik and Udupa, 2004).

Bioevaluation of transdemal patches

The results of drug release studies using the agar plate diffusion method are presented in Figure 1. Compared with the commercially available gentamicin sulphate cream, all batches of the transdermal patches gave great zone of inhibition against *S. aureus, E. coli*, *S. typhi* and *P. aeruginosa*, while *K. pneumoniae* was mildly susceptible to the formulations. From the results presented in Figure 1, the patch formulations gave these zones of inhibition in decreasing order of magnitude: PL 32>PLGA>PDL 04>PDL 05 against *E. coli*, PL 32>PDL 05>PLGA>PDL 04 against *S. typhi*, PL 32>PDL 04>PLGA>PDL 05 against *S. aureus*, PL 32>PDL 05>PDL 04>PLGA against *K. pneumonia*, and PL 32> PDL 04 > PDL 05 > PLGA against *P. aeruginosa*. Compared with the rest of the batches of the formulations, PURASORB® PL 32 gave the greatest zone of inhibition against all the transdermal patches employed in the study. While PURASORB® PDL 04 gave the least zone of inhibition against *S. typhi*, PURASORB® PDL 05 showed the least inhibition zone diameter against *S. aureus* and *E. coli* whereas PLGA exhibited the smallest zone of inhibition against *K. pneumoniae* and *P. aeruginosa*, respectively.

The microbiological test was performed to establish that gentamicin sulphate did not lose activity during formulation and after short-term storage, and was done one month after preparation. The determination of inhibition zone diameter (IZD) using agar plate method was based on the diffusion of an antibiotic agent or

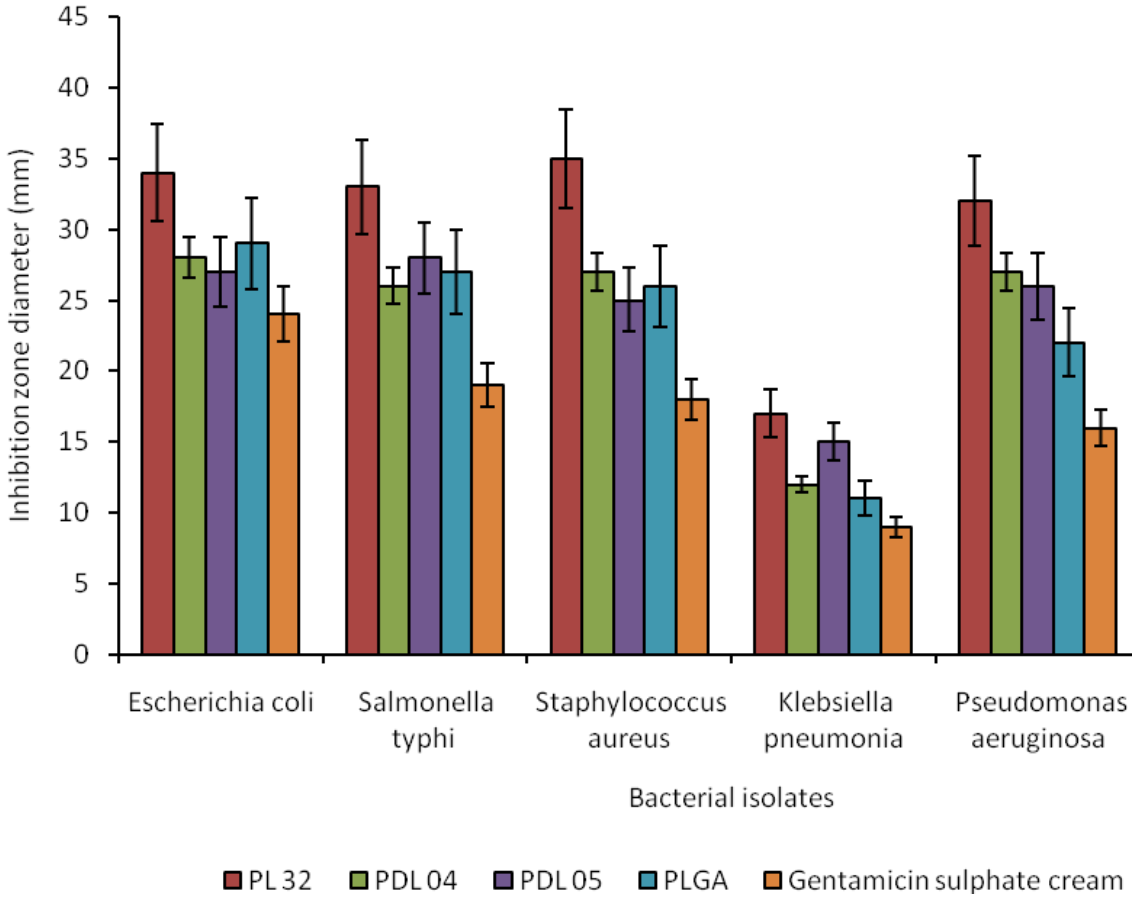

Figure 1. Gentamicin release from drug-loaded transdermal patches as a function of inhibition zone diameter using the bacterial isolates obtained after 1 month of storage (n=3). PL 32, PDL 04, PDL 05 and PLGA are gentamicin-loaded patches containing PURASORB® PL 32, PDL 04, PDL 05 and PLGA, respectively.

formulation thereof through a solidified nutrient agar (Umeyor et al., 2011). From the results presented in Figure 1, the transdermal patches released gentamicin which showed higher IZD compared with the commercially available gentamicin sulphate cream against *E. coli, S. typhi, S. aureus* and *P. aeruginosa*, while *K. pneumoniae* was mildly susceptible to the formulations. Patches of PURASORB® PL 32 showed the best bioactivity. The results indicate that gentamicin loaded into transdermal patches produced very significant zones of inhibition against the Gram positive organism (*S. aureus*) and Gram negative organisms (*S. typhi*, *E. coli*, *S. typhi*, and *P. aeruginosa*) used in the study. The release of gentamicin from the patches depended on the type of polymer since the results showed that batches formulated with PURASORB® PL 32 gave the greatest zones of inhibition against most of the isolates compared with the rest of the formulations. It is discernible from Figure 1 that the biodegradable polyesters, especially PURASORB® PL 32, enhanced the release of gentamicin and therefore are good carrier for its transdermal delivery (Valenta and Auner, 2004).

Stability study of transdermal patches

Figure 2 shows the drug content of the formulations after storage for one year. It is always very important to assess the stability of novel formulations. Stability could be viewed from the degradation of the active ingredients or physical property of the formulation (Panigrahi et al., 2005; Umeyor et al., 2012). In order to determine the change in drug content on storage, stability study was carried out. The results of the stability studies showed that the content of gentamicin in the formulations was not significantly changed on storage, as is evident from Figure 2. The result indicates that the formulations were stable on the required storage condition. In other words, there was a non significant change in the content of gentamicin in the formulations based on the required

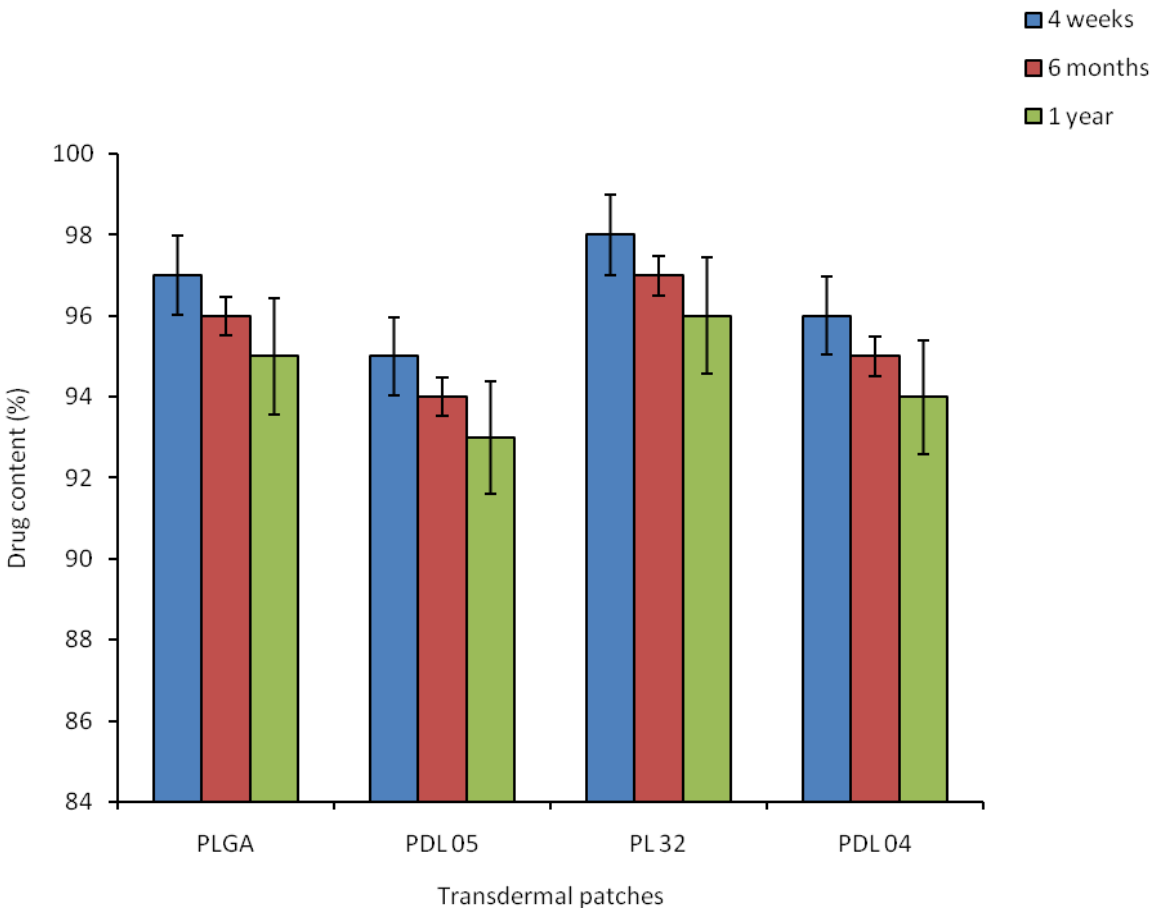

Figure 2. Content of gentamicin in the transdermal patches after storage for one year (n=3). PL 32, PDL 04, PDL 05 and PLGA are gentamicin-loaded patches containing PURASORB® PL 32, PDL 04, PDL 05 and PLGA, respectively

storage conditions.

Conclusion

Polymeric and/or lipid carriers could be exploited to improve the delivery of therapeutic molecules. In this study, gentamicin-loaded transdermal patches based on biodegradable polyesters were successfully prepared by the solvent evaporation technique, which is simple, cheap and reproducible. All the formulations showed good stability, tolerability on rat skin and physicochemical properties. The isolates of *E. coli*, *S. aureus*, *P. aeruginosa*, *S. typhi* and *K. pneumonia* were susceptible to gentamicin incorporated into the transdermal patches. In other words, the antibacterial properties of the drug was retained, with PURASORB® PL 32 patches exhibiting the best bioactivity compared with the commercially available gentamicin sulphate cream that gave the least. It follows that this delivery system (PURASORB® PL 32 patches) could offer a better and more promising approach for the treatment of topical infections caused by gentamicin-susceptible micro-organisms than the commercially available topical gentamicin sulphate cream.

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ABBREVIATIONS

TDDS, transermal drug delivery systems; **PL 32**, **PDL 04**, **PDL 05** and **PLGA** are gentamicin-loaded patches containing PURASORB® PL 32, PDL 04, PDL 05 and PLGA respectively; **APIs**, active pharmaceutical ingredients; **IZD**, inhibition zone diameter; **DSC,** differential scanning calorimetry.

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Full Length Research Paper

Protective effects of aqueous extract of *M. pruriens* Linn. (DC) seed against cisplatin induced oxidative stress and nephrotoxicity in rats

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In the present study, we investigated the effect of the aqueous extract of *Mucuna pruriens*, against cisplatin induced oxidative stress and nephrotoxicity in rats. Nephrotoxicity was induced by a single dose of cisplatin (5 mg/kg body weight *i.p.*). Cisplatin administration resulted in significant increases in urine volume, serum creatinine and urea and significant decrease in creatinine clearance and urinary sodium in comparison with control. Also, the renal tissue from the cisplatin treated rats showed significant decreases in the kidney glutathione content, superoxide dismutase and catalase activity and a significant increase in lipid peroxides levels. Seven days after *M. pruriens* extract at a dose of 200 and 400 mg/kg plus cisplatin treatments significantly decrease urea, creatinine and significantly increase creatinine clearance levels as compared to cisplatin rats in a dose dependent manner. In addition, *M. pruriens* prevented the rise of lipid peroxides and the reduction of superoxide dismutase, catalase and glutathione activities in a dose dependent manner. These results suggest that *M. pruriens* extract has protective effects against cisplatin induced oxidative stress and nephrotoxicity in rats.

Key words: *Mucuna pruriens*, cisplatin, lipid peroxidation, free radicals.

INTRODUCTION

Cisplatin (*cis*-diamminedichloroplatinum II, CP), one of the most potent and widely used anticancer drugs containing platinum, is highly effective against many tumors, including testicular, small cell lung, head and neck, and bladder carcinomas (Meyer et al., 1994). However, the clinical usefulness of this drug is limited by the development of nephrotoxicity, a side effect that may be produced in various animal models (Kim et al., 1997; Greggi et al., 2001; Chirino et al., 2004; Weijl et al., 2004). The xenobiotic-induced alterations in kidney functions are characterized by signs of injury, such as changes in urine volume, creatinine clearance, in glutathione (GSH) status, increase of lipid peroxidation (LPO). Formation of free radicals, leading to oxidative stress, has been shown to be one of the main pathogenic mechanisms of these toxicities and side effects of nephrotoxicants (Greggi et al., 2000; Atessahin et al., 2003). CP induced nephrotoxicity is also closely associated with an increase in LPO in the kidney tissues. This antitumoural drug causes generation of reactive oxygen species (ROS), such as superoxide anion and hydroxyl radical, to deplete of GSH levels and to inhibit the activity of antioxidant enzymes in renal tissue. ROS may produce cellular injury and necrosis via several mechanisms including peroxidation of membrane lipids, protein denaturation and DNA damage (Kim et al., 1997; Mora et al., 2003). *Mucuna pruriens* Linn. DC. (Leguminosae) is annual climbing legume endemic in India and in other parts of the tropics including Central and South America. In India, the plant is known by different local names like ‘‘the cowhage’’, ‘‘velvet’’ bean and ‘‘atmagupta’’. In Ayurvedic system of medicine, *M.*

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pruriens was used for the management of male infertility, nervous disorders and also as an aphrodisiac agent (Pandey et al., 1996; Muthu and Krishnamoorthy, 2011). *M. pruriens* seed powder contains high amount of L-DOPA, a potentially neurotoxic agent used in the treatment of parkinson's disease (Pant et al., 1970). *M. pruriens* seed in addition to levodopa, contains tryptamine, 5-hydroxytryptamine (5-HT), mucunine, mucunadine, prurienine and prurieninine (Mehta et al., 1994).

It is also rich in fatty content (Panikkar et al., 1987). Alcoholic extract of seed inhibit iron-induced lipid peroxidation (Tripathi et al., 2002). *M. pruriens* seed extract has been reported to attenuate progression of renal damage in streptozotocin-induced diabetic mice (Grover et al., 2001). In type 1 and type 2 diabetes, renal production of dopamine was reduced (Carranza et al., 2001) and this reduction was associated with an increase in total body sodium and impaired ability to excrete sodium load (Segers et al., 1996). In light of above objective, current investigation was to study effect of *M. pruriens* seed extract in cisplatin induced nephrotoxicity and oxidative stress damage in rats.

MATERIALS AND METHODS

Plant Extract

The seeds of *Mucuna pruriens* (L.) DC. (MP) were purchased from the United Chemicals and Allied Products, Kolkata, India. It was authenticated by Dr. B. C. Patel, Botany Department, Modasa, India. A voucher specimen was retained in our laboratory for further reference. For the extract, the seeds were powdered in a mechanical grinder. 1 kg seed powder of *M. pruriens* was initially defatted with 750 ml of petroleum ether (60-80°C) then aqueous extract was prepared by cold maceration method in that extract was shaken intermediately and $CHCl_3$ was added to prevent bacterial growth. After seven days, the extract was filtered using Whatman filter paper (No. 1) and then concentrated in vacuum and dried. The yield was 10.05 % w/w with respect to dry powder.

Standardization of extract

Standardization of extract was carried out by high performance thin layer chromatography. The samples were spotted in the form of bands with a Camag microlitre syringe on a precoated silica gel plates 60 F_{254} (20 cm ×10 cm with 0.2 mm thickness, E. Merck, Darmstadt, Germany) using a Camag Linomat V Automatic Sample Spotter (Muttenz, Switzerland).

The plates were prewashed by methanol and activated at 60°C for 5 min prior to chromatography. The plate was developed in a solvent system (6.0 ml) of n-butanol-acetic acid-water (4.0 + 1.0 + 1.0, v/v) in a CAMAG glass twin–through chamber (10 x 10 cm) previously saturated with the solvent for 30 min (temperature 25 ± 2°C, relative humidity 40%). The development distance was 8 cm. Subsequent to the scanning, TLC plates were air dried and scanning was performed on a Camag TLC scanner III in absorbance mode at 280 nm and operated by Win Cats software. Evaluation was via peak areas with linear regression. Calibration curve of standard L-dopa was plotted and was found to be linear in the range of 10-120 µg/ml.

Experimental animals

Sprague Dawely rats weighing 200-250 g were used for the study. The animals were housed in a group of 3 rats per cage under well-controlled conditions of temperature (22 ± 2°C), humidity (55 ± 5%) and 12 h/12 h light-dark cycle. They were maintained under standard environmental conditions and were fed a standard rat chow diet with water given *ad libitum.* The study was approved by Institutional Animal Ethical Committee, Shri B. M. Shah College of Pharmaceutical Education and Research, Modasa, Gujarat, India (IAEC/BMCPER/02/2005-06).

Treatment protocols

The rats were divided into five groups; each group containing six rats. CP was injected to animal intraperioneally at the dose of 5 mg/kg, which is well documented to induce nephrotoxicity in rats (Shimeda et al., 2005; Tebekeme and Prosper, 2007). Group 1 served as control. Group 2 received MP extract (400 mg/kg, p.o.). Group 3 received a single dose CP (5 mg/kg, i.p.). Group 4 received MP extract (200 mg/kg, p.o.) for 6 consecutive days after CP injection. Group 5 received MP (400 mg/kg, p.o.) extract for 6 consecutive days after CP injection. On day 7 after CP injection, blood samples were collected from the tail vein and allowed to clot for 30 min at room temperature. Blood samples were centrifuged at 3000 rpm for 20 min. Serum was separated and stored at -20°C until analysis was done. Serum samples were analyzed spectrophotometrically for urea and creatinine (Bayer Diagnostics Kit, India). The kidneys were removed, washed with ice-cold saline and homogenized in ice cold 0.1 M Tris-HCl buffer (pH 7.4). The homogenate was centrifuged and supernatant was used for the assay of lipid peroxidation (Ohkawa et al., 1979), superoxide dismutase (Mishra et al., 1972), catalase (Aebi et al., 1974), glutathione (Beutler et al., 1963) and total protein estimation (Lowry et al., 1951). The changes in urinary volume were measured at 12 h intervals and the changes in the body weight were also determined throughout the experiments. Urinary sodium was measured by flame photometry. Creatinine clearance was measured according to Jaiswal et al. (1995).

Statistical analysis

Results were expressed as mean ± standard error of mean (S.E.M.). Result were analyzed statistically using analysis of variance (ANOVA) followed by Tukey's test. Values of $p< 0.05$ were considered significant.

RESULTS

Standardization of extract

The concentration of L-dopa in aqueous extract of *M. pruriens* was found to be 5.6%. Comparison of absorption spectrum of the band in the sample track with that of standard L-dopa at R_F 0.39 by overlaping confirmed the presence of L-dopa in the sample and it was found to be one of the major components (Figure 1).

Effect on body weight and urine volume

Rats which received CP showed a marked decrease in body weight, urinary sodium and increase urine volume

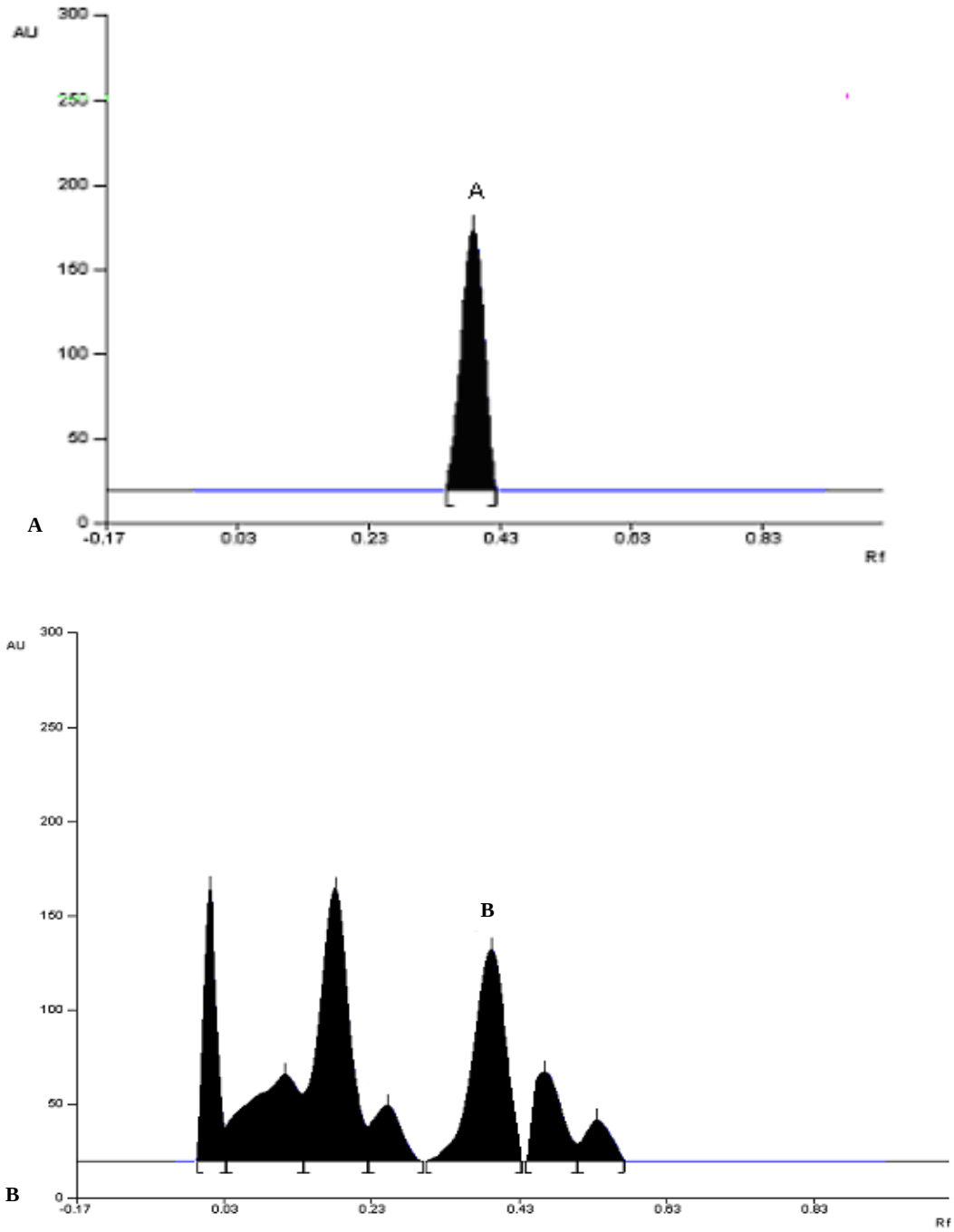

Figure 1. Densitograms of (A) Standard L-dopa (B) a typical seed extract of *Mucuna pruriens* (*Rf* value = 0.39).

as compared to control rats. Treatment with aqueous extract at a dose of 200 and 400 mg/kg did not show any significant change in body weight as compared to CP treated rats. *M. pruriens* seed extract at a dose of 400 mg/kg significantly increase urine volume and urinary sodium as compared to CP rats. However, increase in urine volume and urinary sodium at 200 mg/kg of aqueous extract of *M. pruriens* was not significant (Table 1).

Effect on serum urea, creatinine and creatinine clearance

Cisplatin treated rats showed a significant increase serum urea, creatinine and decrease creatinine clearance levels as compared to control rats. Aqueous extract significantly decrease serum urea and creatinine levels and increase creatinine clearance levels as compared to CP treated rats in a dose dependent manner (Table 1).

Table 1. Effects of aqueous extract of *M. pruriens* on various parameters in cisplatin induced nephrotoxicity in rats.

Parameter	Control	MP 400 mg/kg	CP	CP +MP 200 mg/kg	CP +MP 400 mg/kg
Body weight (g)	223 ± 10.06	225 ± 9.98	185 ± 4.46*	206 ± 6.99	212 ± 8.44
Urine volume (ml/12h)	3.04 ± 0.27	3.18 ± 0.32	16.60 ± 1.06	20.52 ± 1.65	27.98 ± 1.75**
Urinary sodium (mg/ml/12hr)	4.78 ± 0.22	4.79 ± 0.28	1.98 ± 0.33	2.54 ± 0.38	3.93 ± 0.43**
Urea (mg/dl)	40.90 ± 2.99	39.74 ± 3.44	85.80 ± 6.11*	61.94 ± 5.22**	49.78 ± 6.01**
Creatinine (mg/dl)	0.88 ± 0.08	0. 87 ± 0.06	1.90 ± 0.08*	1.22 ± 0.07**	1.00 ± 0.08**
Creatinine clearance (ml/min)	0.60 ± 0.03	0.62 ± 0.03	0.26 ± 0.02*	0.40 ± 0.03**	0.56 ± 0.02**

Each value is mean ± S.E.M. (n = 6); *, Significantly different from normal control, $p<0.05$; **, significantly different from cisplatin control, $p<0.05$.

Table 2. Effects of aqueous extract of *M. pruriens* seed extract on lipid peroxidation and antioxidant parameters in cisplatin induced nephrotoxicity in rats.

Parameter	Control	MP 400 mg/kg	CP	CP +MP 200 mg/kg	CP +MP 400 mg/kg
Lipid Peroxidation (μmole/mg of protein)	0.89 ± 0.11	0.90 ± 0.13	5.84 ± 0.48*	3.91 ± 0.35**	2.37 ± 0.33**
Superoxide dismutase (U/min/mg of protein)	0.53 ± 0.06	0.52 ± 0.05	0.21 ± 0.03*	0.37 ± 0.04**	0.51± 0.04**
Catalase (U/mg of protein)	19.94 ± 2.22	19.78 ± 1.84	9.42 ± 1.01*	16.50 ± 1.77**	19.89 ± 1.72**
Glutathione (μ mole/ mg of protein)	1.53 ± 0.15	1.50 ± 0.17	0.38 ± 0.15*	0.99 ± 0.12**	1.39 ± 0.14**

Each value is mean ± S.E.M. (n = 6); *, Significantly different from normal control, $p<0.05$; **, significantly different from cisplatin control, $p<0.05$.

Effect on lipid peroxidation and antioxidant parameters

Administration of CP in rats produced a significant increase in lipid peroxides and significant decrease in superoxide dismutase, catalase and glutathione levels in kidney tissue as compared to control rats. Treatment with aqueous extract at a dose of 200 and 400 mg/kg significantly decrease lipid peroxides and increase in superoxide dismutase, catalase and glutathione levels in a dose dependent manner as compared to CP treated rats. Aqueous extract alone in rats did not produce any significant change in lipid peroxides and antioxidant parameters in kidney tissue (Table 2).

DISCUSSION

In the present study, the rats treated with CP showed a decrease in body weight. This weight loss was attenuated, but not completely with aqueous extract of *M. pruriens*. Mora et al. (2003) reported suggested that CP induced weights loss might be due to gastrointestinal toxicity and by reduced ingestion of food.

The impairment of kidney function by CP is recognized as the main side effect and the most important dose limiting factor associated with its clinical use. Several investigators reported that the alterations induced by CP in the kidney functions were characterized by signs of injury such as, increase urine volume, urea and creatinine level in serum (Greggi et al., 2001; Naziroglu et al., 2004). In the present study, it was shown that administration of CP to rats increased urine volume, serum creatinine and serum urea and decrease in creatinine clearance and urinary sodium as compared to control one. Treatment with aqueous extract at a dose of 200 and 400 mg/kg significantly decrease serum urea and creatinine level and significantly increase in creatinine clearance in a dose dependent manner. Aqueous extract at a dose of 400 mg/kg also produced a significant increase in urine volume and urinary sodium which was increase in CP treated rats. This effect may be due to presence of L-dopa in the extract because it binds with dopamine receptors. D_1-like receptors are reported

to cause an increase in renal blood flow and glomerular filtration rate, as well as increase in urinary excretion of water and sodium (Hedge et al., 1989; Jose et al., 1992). Several studies have shown the role of dopamine in the regulation of sodium excretion during acute volume expansion and during acute increase in sodium intake (Chen et al., 1991; Hedge et al., 1989; Oates et al., 1979).

The concentration of lipid peroxides as a result of lipid peroxidation shows an increase in CP treated group. The decreased superoxide dismutase activity can cause the initiation and progression of lipid peroxidation in the CP treated rats. This decreased activity may be due to loss of copper and zinc, which are essential for the enzyme activity or reactive oxygen species induced enzyme inactivation (Matsushima et al., 1998). Recent evidences have indicated that the free radicals and reactive oxygen species are involved in the CP induced oxidative stress because of depletion of the GSH concentration and decreased antioxidant enzyme activity in the kidneys (Satoh et al., 2003; Sharma et al., 1985; Zeki et al., 2003). These observations also support the hypothesis that part of the mechanism of nephrotoxicity in the CP treated animals is related to depletion of antioxidants. In the present study, treatment with aqueous extract of *M. pruriens* at a dose of 200 and 400 mg/kg significantly decrease lipid peroxides and significantly increase the superoxide dismutase and catalase levels as compared CP treated rats in dose dependent manner. Rajeshwar et al. (2005) have reported that alcoholic extract of seed of *M. pruriens* inhibit lipid peroxidation *in vitro*. Alcoholic extract of the seeds of *M. pruriens* has anti lipid peroxidation activity which is mediated through removal of superoxide radical and hydroxyl radical (Tripathi et al., 2002). Aqueous extract of *M. pruriens* contain L-dopa which was reported to decrease free radical generation in various *in vitro* radical scavenging models (Gulcin, 2007).

One of the most important intracellular antioxidant systems is the glutathione redox cycle. GSH is one of the essential compounds for maintaining cell integrity because of its reducing properties and participation in the cell metabolism. The depletion in the renal GSH level has been observed in rats in response to oxidative stress caused by CP treatment (Kim et al. 1997; Silva et al., 2001). On the other hand, results of some investigators showed that the kidney damage caused by CP is not associated with decreased in renal GSH (Greggi et al., 2000) or may causes increase in GSH levels (Mora et al., 2003). The mechanism of this antitumoural drug induced change in renal GSH level is not completely understood. However, GSH may modulate metal reduction and the thiol portion is very reactive with several compounds, mainly with alkylating agents such as CP. In this study, GSH levels in the renal tissue of rats treated with CP were lower than normal control group. On the other hand, an increase in GSH levels in the renal tissue indicates that treatment with aqueous extract of *M. pruriens* was caused in response to oxidative stress.

Conclusion

In conclusion, our data suggests that aqueous extract of *M. pruriens* protect the CP induced oxidative stress and nephrotoxicity in rats. The mechanism may be attributed to its free radical scavenging property of L-dopa in the extract.

ABBREVIATIONS

CP, Cisplatin (*cis*-diamminedichloroplatinum II, CP; **GSH,** glutathione; **LPO,** lipid peroxidation; **ROS,** reactive oxygen species; **5-HT,** 5-hydroxytryptamine.

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Full Length Research Paper

Biological screening of extracts of Brazilian Asteraceae plants

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Natural products are a very productive source of leads for the development of medicines. Seven Brazilian Asteraceae adult plants were randomly chosen. The current study was designed to evaluate the antiprotozoal and cytotoxic activities *in vitro* of 21 extracts obtained. Phytochemical properties of the most active extracts also were checked. Cytotoxic activity was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method against human tumor cell lines (HCT-116, OVCAR 8 and SF-295). The antiprotozoal activity was evaluated against *Trichomonas vaginalis*, *Entamoeba histolytica* and *Giardia lamblia.* None of the extracts showed antiprotozoal activity. However, 76% of the extracts displayed moderate to high *in vitro* cytotoxic activities against human cancer cell which suggests that they are a promising source of anticancer compound, since none of the extracts showed hemolytic activity. Terpenoids, flavonoids, saponins, tannins, besides other compound classes, were identified and may be responsible for their antitumor activity. Cytotoxic assays indicate the anticancer potential of Asteraceae species from Brazil.

Key words: Drug prospecting, Antiprotozoal agents, Cytotoxicity, Drug screening assays, Brazilian plant.

INTRODUCTION

Currently, there still persists many difficulties and challenges in cancer therapy such as drug resistance, toxicity and low specificity of drugs (Mesquita et al., 2009). Plants have a long history of use in the cancer treatment. Active constituents of *Catharanthus roseus* (L.) G.Don (Apocynaceae), *Angelica gigas* Nakai (Apiaceae), *Podophyllum peltatum* L. (Berberidaceae), *Taxus brevifolia* Nutt. (Taxaceae), *Ochrosia elliptica* Labill. (Apocynaceae), and *Camptotheca acuminata* Decne. (Cornaceae) have been used in the treatment of advanced stages of various malignancies (Patel et al., 2009). Over 50% of drugs used in clinical trials for anticancer activity were isolated from natural sources or are related to them (Majumdar, 2012). The three anaerobic protozoa, *Entamoeba histolytica*, *Giardia lamblia* and *Trichomonas vaginalis* are highly prevalent

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human-infective parasites with a worldwide prevalence (Cantillo-Ciau et al., 2010). The most effective and commonly used drug in the treatment of these three protozoans is metronidazole. However, this substance has unpleasant side effects such as a metallic taste, headache, dry mouth, urticaria, pruritus, and dark-colored urine (Pérez et al., 2012). Due to these undesired side effects and taking into account the possibility of the development of resistant strains of the *T. vaginalis, E. histolytica*, and *G. lamblia* against metronidazole, there is a clear need for new, effective, and safer antiprotozoal agents.

Natural products, especially of plant origin, represent an excellent starting point for research. In traditional medicine there are also several plants used to treat vaginitis (Girón et al., 1988) and amoebic dysentery (Bautista et al., 2011). Amaral et al. (2006) described 153 plant species from 69 families that were evaluated for their giardicidal activity. It was found that the majority of extracts and fractions obtained from plant species employed in popular medicine for the treatment of diarrhea and dysentery exhibited *in vitro* giardicidal activity, and these were mainly from species belonging to the Asteraceae family.

Asteraceae is the largest family of angiosperms and it comprises 1535 genera and about 23 thousand species distributed in 3 subfamilies and 17 tribes (Bremer, 1994). The plants of the Asteraceae family are very common in the open formations of Brazil, mainly in the *cerrado*, where the family is well represented by approximately 250 genera and 2000 species (Guimarães et al., 2012). Asteraceae species have been used in the Brazilian folk medicine for several therapeutic purposes. For example, species of the genus *Lychnophora*, popularly known as "arnica", are widely used in Brazilian folk medicine as anti-inflammatory, to treat bruise, pain, rheumatism and for insect bites (Ferrari et al., 2012). Species of the genus *Mikania*, known as "guaco", they are widely used in Brazil in the formulation of syrups for the treatment of the respiratory system (Guimarães et al., 2012). Among the native plants of Brazil, species of genus *Baccharis*, popularly known as "carqueja", has been used as diuretic, tonic, digestive, protective and stimulate of the liver, antianemic, anti-rheumatic, obesity control, diabetes, hepatitis and gastroenteritis (Morais and Castanha, 2011).

Aiming to explore the rich Brazilian biodiversity, we initiated a bioprospection of plants from the Asteraceae family occurring in the state of Minas Gerais, Brazil, by screening plant extracts for cytotoxic and antiprotozoal activities.

MATERIALS AND METHODS

Plant material

Seven plants belonging to the Asteraceae family were collected in Ouro Preto-MG, Brazil (April 2010 to April 2012), and were identified by comparison with voucher specimens present in the herbarium, previously identified. Voucher specimens for each plant collected were deposited at the Herbarium José Badini, Universidade Federal de Ouro Preto-UFOP (Table 1).

Extract preparation

Approximately 4 g of the powdered aerial plant material of each specimen was extracted at room temperature by maceration with hexane (100 ml, 3 consecutive extractions over 24 h) followed by extraction using ethyl acetate (100 ml, 3 consecutive extractions over 24 h) and ethanol (100 ml, 3 consecutive extractions over 24 h). The colored solution from each of the plant material was filtered and finally concentrated by vacuum evaporation. The concentrated extract obtained was preserved for further use.

Antiprotozoal activity

E. histolytica, strain HM1:IMSS (ATCC 30459), *T. vaginalis*, strain JT, were maintained in YI-S medium (Diamond et al*.,* 1995) and *G. lamblia*, strain Portland (ATCC 30888), was grown in Diamond's modified TYI-S-33 medium (Diamond et al*.,* 1978). All protozoans were placed in individual vials containing axenic trophozoites cultures (2.4×10^5 *E. histolytica*, 6×10^4 *T. vaginalis* and 1.2×10^5 *G. lamblia* inoculums) in log growth phase.

The extracts were dissolved in 1 ml of dimethylsulfoxide (DMSO). Aliquots of 300 µl of each solution were diluted in 5 ml of culture medium and added in the glass tubes (13 × 100 mm) containing trophozoites, reaching a final concentration test of 17 µg/ml in a final volume of 6 ml.

The vials were incubated for 48 h at 37°C. All assays were performed in triplicate and repeated twice. Three vials were used as negative control (inoculum + medium) and three as positive control (Metronidazole, Sigma-Aldrich®). Protozoans viability was qualitatively measured using an inverted microscope (Nikon TMS), to observe trophozoites motility and adherence by comparing with the positive and negative controls.

Cytotoxic assay

The antiproliferative potential of the seed extracts was evaluated by the MTT assay (Mosmann 1983) against 3 human tumor cell lines: HCT-116 (colorectal carcinoma), OVCAR 8 (ovarian) and SF-295 (glioblastoma), all obtained from the National Cancer Institute (Bethesda, MD, USA). All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penincillin and 100 µg/ml streptomycin, at 37°C with 5% CO_2. Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide (MTT) to a purple formazan product. Briefly, cells were plated in 96-well plates (0.7×10^5 cells/ml) and extracts (50 µg/ml) were added to each well. After 72 h of incubation, the supernatant was replaced by fresh medium containing MTT (0.5 mg/mL), the formazan product was dissolved in 150 µl DMSO and absorbance was measured at 595 nm (DTX 880 Multimode Detector, Beckman Coulter). Doxorubicin (0.3 µg/ml, Sigma Aldrich) was used as positive control. The results are summarized in Table 1.

Hemolytic test

The hemolytic test was performed in 96-well plates following the method described by Berlinck et al. (1996). Each well received 50 ml of 0.85% NaCl solution containing 10 mM $CaCl_2$. The first well

Table 1. Tumor cell proliferation inhibition (%) of crude extracts of seven plant species belonging to *Brazilian Asteraceae* family determined by MTT assay after 72 h of incubation at the concentration of 50 µg/ml.

Species (Voucher no.)	Extract	Cell proliferation inhibition (%)*		
		HCT-116	OVCAR 8	SF-295
Stevia urticifolia Thunb. (OUPR 24049)	H	63.0 ± 0.5	61.2 ± 0.3	55.7 ± 1.1
	EtAc	97.8 ± 7.4	97.9 ± 0.1	69.6 ± 4.0
	Et	5.2 ± 0.2	4.9 ± 1.4	41.8 ± 1.1
Vernonia polyanthes Less. (OUPR 26355)	H	17.6 ± 0.9	10.9 ± 1.9	53.5 ± 2.6
	EtAc	99.4 ± 1.2	92.5 ± 2.7	82.8 ± 0.8
	Et	15.9 ± 2.1	–2.1 ± 2.2	45.6 ± 1.2
Vernonia crotonoides (DC.) Sch.Bip. (OUPR 25896)	H	55.8 ± 5.6	32.6 ± 13.0	36.9 ± 5.2
	EtAc	100.7 ± 0.8	98.6 ± 0.7	89.6 ± 2.8
	Et	7.1 ± 1.1	4.5 ± 0.8	53.1 ± 0.9
Moquinia racemosa DC. (OUPR 26602)	H	58.1 ± 0.5	51.9 ± 2.1 97.8	55.7 ± 1.2
	EtAc	98.3 ± 1.7	0.6	96.9 ± 0.4
	Et	23.3 ± 0.0	5.8 ± 0.3	52.3 ± 0.4
Mutisia campanulata Less (OUPR 26754)	H	47.4 ± 0.5	49.1 ± 0.6	58.3 ± 0.1
	EtAc	3.3 ± 0.9	6.4 ± 0.3	45.3 ± 0.2
	Et	24.2 ± 4.2	5.4 ± 7.1	49.6 ± 0.8
Acanthospermum australe (Loefl.) Kuntze (OUPR 25895)	H	48.7 ± 0.1	75.3 ± 0.7	54.5 ± 6.5
	EtAc	96.5 ± 5.6	96.4 ± 0.0	85.4 ± 0.4
	Et	12.0 ± 2.8	2.2 ± 4.0	42.5 ± 3.0
Calea fruticosa (Gardner) Urbatsch, Zlotsky & Pruski (OUPR 26290)	H	72.7 ± 3.2	95.3 ± 0.5	45.2 ± 0.3
	EtAc	99.4 ± 0.2	96.7 ± 1.0	96.2 ± 2.4
	Et	95.7 ± 19.5	95.6 ± 19.4	61.2 ± 10.9

H, hexane; EtAc, ethyl acetate; Et, ethanol. *Results are expressed as mean ± standard error mean (S.E.M.) from two independent experiments for colorectal carcinoma (HCT-116), ovarian (OVCAR 8), and glioblastoma (SF-295) human cancer cells. All cell lines were plated with RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penincillin and 100 µg/ml streptomycin, at 37°C with 5% CO_2. Doxorubicin (0.3 µg/ml) was used as positive control. High activity: > 75%; moderated activity: 50 to 75%; low activity: < 50 %.

was the negative control that contained only the vehicle (1% DMSO), and in the second well 50 ml of test substance that was diluted in half was added. The extracts were tested at concentrations ranging from 1.56 a 200 µM. The last well received 50 ml of 0.2% triton X-100 (in 0.85% saline) to obtain 100% hemolysis. Then, each well received 50 ml of a 2% suspension of mouse or human erythrocytes in 0.85% saline containing 10 mM $CaCl_2$. After incubation at room temperature for 1 h, and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectroscopically as absorbance at 540 nm.

Phytochemical screening

Chemical tests were carried on the most active extracts (that exhibited high *in vitro* cytotoxic activities, cell growth inhibition between 75 to 100%) to identify the phytoconstituents, that is, alkaloids, flavonoids, saponins, tannins, and terpenoids, as per the standard procedure (Edeoga et al., 2005; Egwaikhide and Gimba, 2007; Abalaka et al., 2011). The results are summarized in Table 2.

Statistical analysis

The analysis of cell proliferation (*in vitro* cytotoxic assays) and hemolytic potential were determined by non-linear regression using the Graphpad program (Intuitive Software for Science, San Diego, CA).

RESULTS

Table 1 summarizes the cytotoxic activities displayed by the Asteraceae plant extracts that were evaluated in this research. Of the 21 extracts tested, the analyses by MTT assay showed that 16 (76%) displayed moderate to high *in vitro* cytotoxic activities against human cancer cells. Ethyl acetate extracts were the most active against the 3

Table 2. Phytochemical analysis of the most active extracts.

Species	Extract	Terpenoid	Flavonoid	Saponin	Alkaloid	Tannin
S. urticifolia	EtAc	+	+	+	–	–
V. polyanthes	EtAc	+	+	–	–	–
V. crotonoides	EtAc	+	+	–	–	–
M. racemosa	EtAc	+	+	+	–	–
A. australe	H	+	+	–	–	–
A. australe	EtAc	+	+	–	–	–
C. fruticosa	H	+	–	–	–	–
C. fruticosa	EtAc	+	+	–	–	–
C. fruticosa	Et	–	+	+	–	+

H, Hexane; EtAc, ethyl acetate; Et, ethanol. Key: + = present ; – = absent

human tumor cell lines when compared to those from hexane or ethanol. *Calea fruticosa* (Gardner) Urbatsch, Zlotsky and Pruski was the only plant species that revealed promising *in vitro* cytotoxic action with their hexane, ethyl acetate and ethanol extracts. All extracts were found to be ineffective against all the tested protozoa. The results of phytochemical screening of the most active extracts are shown in Table 2. Alkaloids were not present in any extract. The presence of tannins was observed only in *C. fruticosa* (ethanolic extract). The presence of terpenoids has been detected in all extracts except for the ethanolic extract of *C. fruticosa*. Flavonoids were also observed in all extracts except for the hexanic extract of *C. fruticosa*. Only *Stevia urticifolia* Thunb. (ethyl acetate extract), *Moquinia racemosa* DC. (ethyl acetate extract) and *C. fruticosa* (ethanolic extract) showed the presence of saponins.

DISCUSSION

The 16 extracts that showed moderate to high *in vitro* cytotoxic activities against human cancer cells were considered promising anticancer compound sources. Researches for antineoplasic compounds have demonstrated the great pharmacological relevance of the plant extracts (Ferreira et al., 2011). According to the American National Cancer Institute, the limit to be considered a promising crude extract for further purification is a value lower than 50 µg/ml and cell proliferation inhibition is higher than 90% (Suffness and Pezzuto, 1990; Ferreira et al., 2011). In relation to the cytotoxic or antitumor activity of these plant species, rare findings are available. For example, *Acanthospermum australe* extracts were capable to increase the survival of Ehrlich ascites tumor-bearing mice and stimulated myelepoiesis, which can influence on antitumor immune responses (Mirandola et al., 2002).

Studies indicate that some plant substances like polyphenols, epicatechins, steryl glycosides and triterpenoid saponins cause damage to red cell membranes and produce hemolysis (Costa-Lotufo et al., 2002). The mechanical stability of erythrocyte membrane a good indicator of insults by vegetal substances (Sharma and Sharma, 2001; Santos et al., 2010). Then, hemolysis detection is an useful and cheap technique which can displays the effect of increasing concentrations and can to be sigmoidally related to the logarithm contact time, emphasizing the membrane stability as a biological complex to maintain its structure under stress conditions, such as oxidation, hipotonicity, pH changes, heat and in presence of osmotic active solutes (Van Ginkel and Sevanian, 1994; Sharma and Sharma, 2001; Freitas et al., 2008). Herein, none of the extracts tested caused hemolysis even at the highest concentration (200 µg/ml), suggesting that the mechanism of cytotoxicity is probably related to a more specific pathway. Guo and Gao (2013) described the antiproliferative effects of SPV (total saponin extract from *Patrinia villosa*) and FPV (total flavonoid extract from *P. villosa*) on four cancer cell lines and concluded that the mechanisms involved in cancer chemoprevention by FPV and SPV extracts were cell cycle arrest and induction of apoptosis. Targeting cell cycle and apoptotic pathways has emerged as an attractive approach for treatment of cancer (Aslantürk and Çelik, 2013).

Vernonia polyanthes Less., known as assa-peixe, have been studied and their pharmacological properties which include antinociceptive and anti-inflammatory (Temponi et al., 2012), antibacterial (Silva et al., 2012), antifungal and leishmanicidal (Braga et al., 2007), have been established. Fixed acids, alkaloids, aminoacids, coumarins, steroids, triterpenes, anthraquinones, flavonoids, saponins and tannins were detected in infusions of *V. polyanthes* and may be responsible for their pharmacological effects (Temponi et al., 2012). *Acanthospermum australe* (Loefl.) Kuntze is an annual shrub widely distributed in South America. In Brazil, where it is popularly known as “carrapichinho” or “carrapicho-de-carneiro”. Its aerial parts are used in folk medicine as a tonic, diaphoretic, eupeptic, vermifuge, antidiarrheal, antimalarial, antigonorrheal, febrifuge, and antianemic

(Lorenzi and Matos, 2002). Previous phytochemical investigations of *A. australe* have led to the isolation of germacranolides, melampolides, diterpene lactones and 6-methoxyflavonoids (Bohlmann et al., 1979, 1981; Matsunaga et al., 1996). Antiviral (Rocha Martins et al., 2011) and antitumor properties (Mirandola et al., 2002) were already described for this species of plant. *Vernonia crotonoides* (DC.) Sch.Bip. is a synonym of *Eremanthus crotonoides* (DC.) Sch.Bip. (Robinson, 1999). Bohlmann et al. (1982) revealed that germacrene D, bicyclogermacrene, α-humulene, caryophyllene, lupeol and its acetate derivative, taraxasterol and its acetate, stigmasterol, and sesquiterpene lactones are present in aerial parts of *E. crotonoide*s. Lobo et al. (2012) evaluated the antiproliferative effects of extracts and sesquiterpene lactone from *E. crotonoides* against two brain tumor cell lines. Dichloromethane fraction was cytotoxic to both glioblastoma multiforme cell lines. Centratherin alone was also evaluated against both U251 and U87-MG cells, which showed IC_{50} values comparable with those obtained for the commercial anticancer drug doxorubicin.

To our knowledge, no further research was carried out with the species *S. urticifolia, M. racemosa*, *Mutisia campanulata* Less, and *C*. *fruticosa*.

In this study, the evaluation of the extracts (hexane, ethyl acetate and ethanol) against *E. histolytica*, *T. vaginalis* and *G. lamblia*, was carried out. However, none of the extracts showed antiprotozoal activity.

The Asteraceae plant species tested showed important activity against human tumor cell lines examined. These findings are the base for further studies to isolate (guided by biological assays) and elucidate, the structure of the bioactive compounds assessed from these plants.

Our results are a contribution to a better understanding of the Brazilian biodiversity, which indicate that these natural sources may become an important source for therapeutic agents.

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