BJPS

Brazilian Journal of Pharmaceutical Sciences
vol. 49, n. 4, oct./dec., 2013

Article

Croton schiedeanus Schltd prevents experimental hypertension in rats induced by nitric oxide deficit

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Croton schiedeanus Schltd (N.V.: "almizclillo") is a plant used in traditional medicine as an antihypertensive in Colombia. It contains flavonoid, diterpenoid and fenilbutanoid metabolites that have vasodilatation effects linked to the NO/cGMP pathway. This work aimed to assess the capacity of a 96% EtOH extract to prevent the hypertension induced by nitric oxide (NO) deficiency in rats. The NO synthase inhibitor L-NAME (10 mg/kg/d, i.p) was administered during five weeks to three groups of rats (6–7 animals): *C. Schiedeanus* (200 mg/kg/d, p.o), enalapril (reference, 10 mg/kg/d, p.o) and vehicle (control: olive oil 1 ml/kg/d, p.o). In addition, the blank group received only vehicle. The arterial blood pressure (BP) and heart rate (HR) were measured daily for six weeks. After sacrificing the animals, the aortic rings were isolated, contraction was triggered with phenylephrine (PE 10^{-6} M) and relaxant responses were achieved with cumulative concentrations of acetylcholine (ACh, 10^{-10} – 10^{-4} M). L-NAME increased the systolic arterial pressure in the control group, attaining mean values of 131 mm Hg at week 5, whereas the *C. schiedeanus,* enalapril and blank groups maintained blood pressure under 100 mm Hg. The capacity of PE to contract aortic rings was greater in the *C. schiedeanus,* enalapril and blank groups than in the control group (2157, 2005, 1910 and 1646 mg, respectively). The pEC_{50} values for ACh were as follows: *C. Schiedeanus* (6.89) >enalapril (6.39) > blank (5.68) > control (5.09). These results give support to *C. Schiedeanus* as a natural antihypertensive source.

Uniterms: *Croton schiedeanus*/pharmacognosy. Almizelillo/use/hypertension prevention. Nitric oxide/deficit. Hypertension/prevention. Plant extracts/vasodilatation effects. Medicinal plants.

Croton schiedeanus Schltd (NV: "almizclillo") é utilizado na medicina tradicional da Colômbia para o tratamento da hipertensão arterial. Outras pesquisas demonstraram que a planta tem metabólitos como os flavonoides, os diterpenoides e os fenilbutanoides, os quais têm comprovados efeitos vasodilatadores vinculados com a via NO/GMPc. O objetivo deste estudo foi avaliar a capacidade do extrato de *Croton schiedeanus* Schltd em EtOH a 96% na prevenção da hipertensão induzida pela deficiência de óxido nítrico (NO), em ratos. O inibidor da NO sintetase L-NAME (10 mg/kg/d, ip) foi administrado durante cinco semanas em três grupos de ratos (6-7 animais): *C. schiedeanus* (200 mg/kg/d, v.o.), enalapril (referência, 10 mg/kg/d, v.o.) e o veículo (controle: azeite de oliva 1 mL/kg/d, v.o.). O grupo branco recebeu somente o veículo. A pressão sanguínea (BP) e a frequência cardíaca (FC) foram medidas diariamente em um período de seis semanas. Após o sacrifício, os anéis aórticos foram isolados e contraídos, utilizando fenilefrina (PE 10^{-6} M) e as respostas para a relaxação foram obtidas com doses acumulativas de acetilcolina (ACh, 10^{-10}-10^{-4} M). Os resultados demonstraram que o L-NAME provocou incremento significativo da pressão nos ratos do grupo controle, obtendo-se valores médios de 131 mm Hg na quinta semana. No entanto, os grupos *C. schiedeanus*, enalapril e branco mantiveram a pressão arterial aos níveis médios iniciais 100 mm Hg. A capacidade da PE para fazer a contração dos anéis da aorta foi maior nos grupos *C. schiedeanus*, enalapril e branco do que no grupo controle (2157, 2005, 1910 and 1646 mg, respectivamente). Os valores de pCE_{50} de ACh foram os seguintes: *C. schiedeanus*

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(6,89) > enalapril (6,39) > branco (5,68) > controle (5,09). Pode-se afirmar que estes resultados dão suporte à *C. schiedeanus* como fonte natural anti-hipertensiva.

Unitermos: *Croton schiedeanus*/farmacognosia. Almizelillo/uso/prevenção da hipertensão. Óxido nítrico/déficit. Hipertensão/prevenção. Extratos vegetais/efeito vasodilatador. Plantas medicinais.

INTRODUCTION

Hypertension or high blood pressure is one of the main health issues in the world. If it is not controlled in an adequate way it can lead to severe consequences such as cardiac failure, renal insufficiency, brain-vascular stroke, heart attack and peripheral vascular disease. It is the primary and most common risk factor for heart disease, stroke and renal disease. One in six people worldwide, or nearly one billion, are affected by high blood pressure and it is estimated that this number will increase to 1.5 billion by 2025 (Kearney *et al.*, 2005). Unlike most diseases, high blood pressure does not present any symptoms and is therefore called the "silent killer". High blood pressure is prevalent in every part of the world, in every region of any nation and in every community (Chockalingam, 2008).

Endothelial dysfunction is one of the main underlying pathophysiological alterations in cardiovascular disorders like hypertension, coronary artery disease and heart failure. Nitric oxide (NO) plays an important role in the maintenance of endothelial integrity. If NO it is produced continuously in the physiological range it helps to protect the endothelium from the challenges that inflammatory molecules carry that induce thrombus, atheroma, apoptosis and smooth muscle cell proliferation (Mizuno *et al*., 2010).

Loss of the capacity to produce NO in an individual is accompanied by enhanced vulnerability to hypertension and related disorders as a result of increased oxidative stress in the vessels and myocardium (Taddei *et al*., 1992; Panza *et al*., 1993; Mason *et al*., 2006). Reduction in NO synthesis or increase in NO degradation leads to a decrease in cyclic guanosine monophosphate (cGMP) formation, which promotes vasoconstriction responses, platelet adhesion and proliferation of vascular smooth-muscle cells, favouring vascular hypertrophy and occlusive vascular disease (Forte, Copland, 1997).

Pharmacological and non-pharmacological measures that protect against endothelia dysfunction help to preserve cardiovascular function. Angiotensin converting enzyme inhibitors, statins and some beta blocking agents are examples of drugs that favour NO production, reduce oxidative stress and ameliorate endothelial lesions (Tzemos *et al*., 2001; Tschöpe *et al*., 2002; Mason *et al*., 2004).

Among medicinal plants that seem to favour the NO/cGMP pathway is *Croton schiedeanus* Schltd, the scientific name of a plant known in Colombia as "almizclillo". Infusions made from the leaves of this species are used in traditional medicine to treat high blood pressure in temperate parts of this country. The vasodilatory effect of ethanol extracts of *C. schiedeanus* has been described previously (Guerrero *et al*., 2001; Guerrero *et al*., 2002a) as well as their major metabolites and relaxant mechanisms related to the NO/cGMP pathway (Guerrero *et al*., 2002b; Guerrero *et al*., 2002c; Correa *et al*., 2008; Carrón *et al*., 2010). However, the capacity of this species to prevent hypertension in rats induced by NO deficit has not yet been examined in an experimental model.

The aim of this study was to assess the effect of a *C. schiedeanus* ethanol extract upon hypertension induced by the NO synthase inhibitor L-NAME (*N*ω-*Nitro*-L-arginine methyl ester hydrochloride) in Wistar rats.

MATERIAL AND METHODS

Extract Preparation

Plant material from *C. schiedeanus* Schltd was collected from the region of Tocaima, Cundinamarca, Colombia in June 2011. Its identity was confirmed by comparison with a specimen (Code No. COL 432164) classified by Dr. Jose Luis Fernandez and kept in the Herbarium of Natural Science Institute at the National University of Colombia. The aerial part (approx. 12 kg of stems and leaves) was dried in an oven with circulating air at 40 ºC during 48 hours. The dried up material was ground and the resulting powder percolated through dripping with 96% EtOH. Afterwards, it was filtered and concentrated in a rotary evaporator under reduced pressure until it was completely dry. For pharmacological trials, the extract was suspended in olive oil to a concentration of 200 mg/mL.

Experimentation animals and treatments

Male Wistar rats (27) were raised in colony cages and exposed to a 12 h dark/light cycle with controlled temperature and moisture (22 ºC, 70%). They were fed a normal laboratory diet with free access to water and food. Cardiovascular experiments were carried out on rats aged

9–11 weeks and weighing 280–320 g. The experimental procedure was approved by the institutional ethics committee. The animals were supplied by the Bioterium of the Pharmacy Department at the National University of Colombia, (UNCSB).

Animals were previously acclimated during two weeks and then randomly assigned to four treatment groups (n=6–7): *C. schiedeanus* plus L-NAME, enalapril plus L-NAME (as reference), vehicle: olive oil plus L-NAME (control), and vehicle without L-NAME (blank group). The dosage regimen was: *C. schiedeanus*, 200 mg/kg/d, p.o. (according to preliminary trials), enalapril, 10 mg/kg/d, p.o. and olive oil 0.1 mL/100 g. L-NAME, 10 mg/kg, i.p. was administered every 48 h to all groups except the blank group from week 1, seven days after *C. schiedeanus*, enalapril and olive oil administration had been started (week 0).

Measurement of Indirect blood pressure (IBP) and heart rate (HR)

Indirect blood pressure and heart rate were measured with a non-invasive method called the "tail cuff device", placing the base of the rat's tail into the light of an ultrasound transducer (*Columbus Instruments 0133-002L, model 59)* capable of capturing the pulse signal and blood pressure. When external pressure is generated, the intensity of the pulse signal disappears due to the occlusion of blood vessels; the signal reappears when occlusion is reduced; this phase of the pulse is considered as the systolic pressure (Van Vliet *et al.,* 2000). The transducer was plugged into a digital analogue Blast (LabTrax WPI) in order to visualize the results using specific software (DataTrax WPI program).

Aortic ring preparation

Male Wistar rats obtained from each experimental group (*C.schiedeanus*, enalapril, blank and control), were anaesthetized with ether and then sacrificed. The descending thoracic aorta was dissected and placed in a petri dish containing an oxygenated Krebs solution with the following composition (mM): NaCl 118.7; KCl 4.7; $CaCl_2$ 2.5; $NaHC0_3$ 25.0; $MgS0_4.7H_20$ 1.2; Glucose 11.0 and ascorbic acid 0.1. The thoracic aorta rings (3–4 mm in length) were carefully excised and submerged in Allhin organ chambers containing 10 mL of Krebs solution bathing medium maintained at 37 ºC, pH between 7.38 and 7.42 ,and continuously gassed with a carbogen mixture of 95% O_2 and 5% CO_2. About 8–10 rings were obtained from each aorta.

Each ring was introduced inside an isolated organ bath containing 10 mL of Krebs solution maintained at 37 ºC with constant carbogenization. The ring was attached with two steel hooks, the inferior anchored to the bath and the upper connected to an isometric force transducer (Fort 10/WPI) coupled to an amplification and digital-analogue conversion system (Bridge 8/IsoDam, LabDataTrax, WPI) for signal analysis in the computer.

A basal tension of 2 g was applied to each preparation with a stabilization period of 60–90 min, during which the Krebs solution was changed every 10–15 min. Once equilibrium was reached, the aortic rings incubated in Krebs solution were exposed to 10^{-6} M FE until the contractile response reached a steady tension (approx. 40 minutes). Afterwards, acetylcholine (ACh) was added cumulatively every 30 s (from 10^{-10} to 10^{-4} M) in aliquots of 0.5 log units of concentration.

Solutions

The following drugs, salts and solutions were used: olive oil (Aceitesublime® G. Sensat), enalapril (Enalapril 5 mg LaFrancol®), *N*ω-nitro-L-arginine methyl ester, phenylephrine (FE), acetylcholine (Ach), propylene glycol, citric acid, sodium citrate, potassium chloride, magnesium sulphate, potassium hydrogen phosphate, EDTA, L-ascorbic acid, sodium chloride, calcium chloride, sodium bicarbonate, and glucose (Sigma®).

Statistical and data analysis

All the results are expressed as mean ± standard mean error (SME). Analysis of variance was performed followed by the minimum significance difference (MSD) test to identify groups responsible for differences in blood pressure, heart rate and ring contraction, assuming $p \leq 0.05$ as significant. Dose–response curves of isolated aortic rings were analysed by a sigmoid curve-fitting analysis to give the negative log of the concentration of ACh producing a 50% in the maximal relaxant response (pEC_{50}). SPSS® 20 and Excel® 2010 programs were used for data analysis.

RESULTS

Effects of *C. schiedeanus* on systolic arterial pressure and heart rate

The mean systolic arterial pressure value at basal conditions (week 0) ranged from 99 to 104 mm Hg (n=27) without significant differences between groups. The increase in arterial pressure induced by L-NAME was significant from week 2 to week 5, attaining values

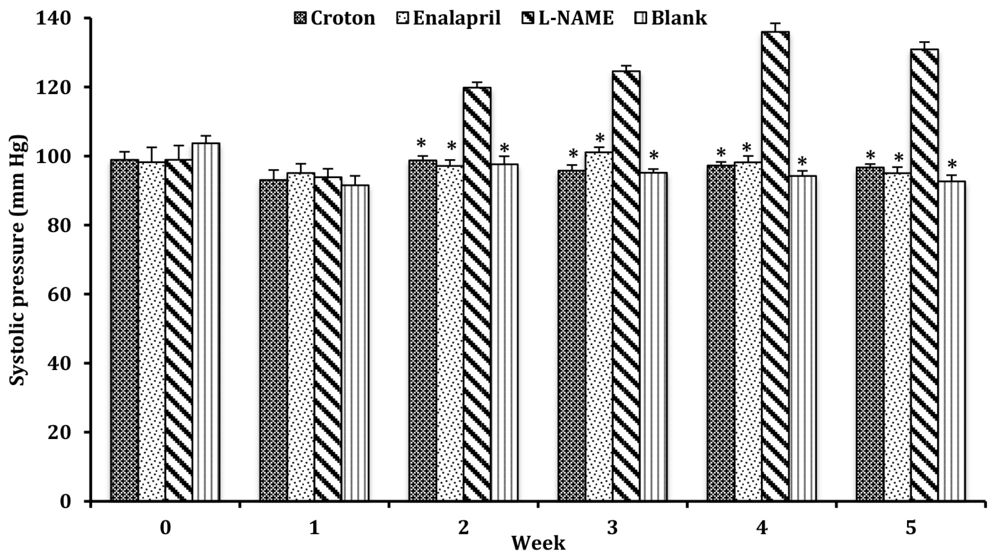

FIGURE 1 - Values of systolic blood pressure in Wistar rats treated with: (1) ethanolic extract of *C. schiedeanus* Schltd (200 mg/kg/day, p.o.) plus L-NAME (10 mg/kg, i.p.), (2) enalapril (reference drug, 10 mg/kg/day, p.o.) plus L-NAME (10 mg/kg, i.p.), (3) vehicle (control, olive oil 0.1 mL/100 g, p.o.) plus L-NAME (10 mg/kg, i.p.) and (4) vehicle (blank, olive oil 0.1 mL/100 g, p.o.). Results are expressed as means ± S.E.M. *p<0.05 with respect to the control group.

of 136 ± 3 mm Hg. *C. schiedeanus* was able to maintain pressure values below 100 mm Hg during all weeks. The effect of the reference drug enalapril was similar to *C.schiedeanus* and to the group of rats not exposed to L-NAME (blank group) (Figure 1). The basal heart rate value was 436±36 ppm. There was a tendency to heart rate decrease in the *C. schiedeanus* and enalapril groups that did not attain statistical significance (data not shown).

In vitro aortic ring studies

Stimulation of aortic rings with FE ($1x10^{-6}$ M) resulted in a sustained contraction, greater in the *C. schiedeanus*, enalapril and blank groups than in the control group (2157±147, 1910±144, 2005±180 and 1646±146 mg, respectively, *n*=39, p>0.05). Cumulative addition of ACh (10^{-10} – 10^{-4} M) in rats previously exposed to L-NAME and treated with *C. schiedeanus* caused a greater relaxant response that reduced the contraction previously induced by FE to 42%. The ACh relaxant response was lower in the enalapril and blank groups (33 and 30%, respectively) and significantly lower in the control group (rats exposed only to L-NAME, 22%, p<0.05) (Figure 2). According to ACh pEC_{50} values, the ACh relaxant potency was as follows: *C. schiedeanus* (6.89) > enalapril (6.39) > blank (5.68) > control (5.09) (Table I).

DISCUSSION

This study shows that a *C. schiedeanus* ethanolic extract is able to prevent the hypertension induced by L-NAME in Wistar rats and, at the same time, improve the vascular relaxant response in isolated aortic rings. Previous works had shown the vasorelaxant effects induced by the active principles in this species, the flavonoid nature of these compounds, the probable synergistic effects induced by them and the importance of the NO/cGMP pathway in their mechanisms of action (Guerrero *et*

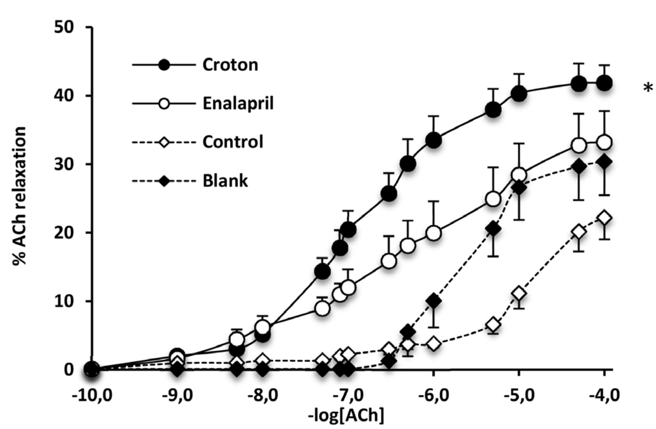

FIGURE 2 - Cumulative (10^{-10}- 10^{-4} M) Acetylcholine dose-response curves in intact aorta rings prepared from Wistar rats previously treated with: (1) ethanolic extract of *Croton schiedeanus* Schltd (200 mg/kg/day, p.o.) plus L-NAME (10 mg/kg, i.p.), (2) enalapril (reference drug, 10 mg/kg/day, p.o.) plus L-NAME (10 mg/kg, i.p.), (3) vehicle (control, olive oil 0.1 mL/100 g, p.o.) plus L-NAME (10 mg/kg, i.p) and (4) vehicle (blank, olive oil 0.1 mL/100 g, p.o.). Results are expressed as means ± S.E.M. *p<0.05 with respect to the control group.

TABLE I - pEC_{50}(-log[EC_{50}]) and % E_{max} values (% of maximal effect) induced by Ach (10^{-10}- 10^{-4} M) in isolated aortic rings contracted with FE (10^{-6} M) from Wistar rats previously treated with: (1) ethanolic extract of *C. schiedeanus* Schltd (200 mg/kg/day, p.o.) plus L-NAME (10 mg/kg, i.p.), (2) enalapril (reference drug, 10 mg/kg/day, p.o.) plus L-NAME (10 mg/kg, i.p.), (3) vehicle (control, olive oil 0.1 mL/100 g, p.o.) plus L-NAME (10 mg/kg, i.p) and (4) vehicle (blank, olive oil 0.1 mL/100 g, p.o.). Results are expressed as means, fiducial limits and S.E.M., respectively. *p<0.05 with respect to the control group

Treatment	pEC_{50}	% E_{max}
L-NAME plus *C. schiedeanus*	6.89 [6.85 – 6.92]*	41.9 ± 2.6*
L-NAME plus enalapril	6.39 [6.37 – 6.41]	33.2 ± 4.6
L-NAME plus vehicle (control)	5.09 [5.00 – 5.18]	22.4 ± 3.2
Vehicle (blank)	5.68 [5.66 – 6.59]	30.4 ± 4.9

al., 2002a; Guerrero *et al*., 2002b; Carrón *et al*., 2010). However, until now, the antihypertensive effect of this species administered p.o, had not been demonstrated in an experimental model of hypertension in rats.

The hypertension induced by L-NAME is a very useful model in searching for potential new drugs for hypertension. Several groups of antihypertensive drugs are active in this model, including: angiotensin converting enzyme inhibitors, angiotensin receptor blockers, calcium channel blockers and some diuretics and beta blocking drugs (Jover, Mimran, 2001; García *et al*., 2006). In addition, this model can show a cardio-protective effect of drugs against cardiovascular remodelling (Massion, Balligand, 2007). This indicates the pivotal role of NO in the physiology of the myocardium and vessels and the importance that alterations in this metabolite play in cardiovascular disorders like hypertension, coronary artery disease and heart failure. In fact, drugs that protect the myocardium and vessels favouring the effect of nitric oxide can reduce the morbidity and mortality of these disorders (von Luederand Krum, 2013). It is interesting to observe that *C. schiedeanus* prevents hypertension in a manner that is not inferior to the reference drug used in this study, enalapril.

The preservation of vascular function obtained with *C. schiedeanus* became apparent examining the effect of ACh in isolated aortic rings from Wistar rats previously treated with this extract and exposed at the same time to a deficit in NO using L-NAME. Whereas the relaxant effect of ACh was reduced in control rats, ACh attained a better relaxant response in rats treated with *C. schiedeanus*, even better than the reference drug or the group not exposed to an NO deficit (blank group). In addition, the vascular contractile capacity tended to increase as the result of previous treatment with *C. schiedeanus*, as has been seen with polyphenols compounds such as quercetin, among others, that increased NO availability (Duarte *et al*., 2001).

The importance of polyphenol compounds for preserving cardiovascular function and their link to the NO/cGMP pathway has recently been reemphasized (Estruch *et al*., 2013; Habauzitand Morand, 2012). This aspect could be especially critical at the beginning of endothelia dysfunction, before a cascade of pathophysiological events that increase the cardiovascular lesion can take place. Foods rich in polyphenol compounds, such as coconut and tea, improve blood pressure and cholesterol levels, reducing endothelial dysfunction and, consequently, the predisposition to plaque formation and thrombus generation. *C. schiedeanus* would represent another rich source of flavonoid compounds that help to protect against disorders related to the progression of endothelial dysfunction.

The relation of the active metabolites from *C. schiedeanus*, mainly the flavonoid compounds ayanin, quercetin 3,7-dimethyl ether and quercetin 3,7-ethyl ether to the NO/cGMP pathway has been described previously (Guerrero *et al*, 2002a; Guerrero *et al*., 2002b). They seem to act in a synergistic fashion, favouring the persistence of NO, with the aim that this metabolite can activate the cascade of the cGMP pathway that ends in vasodilation and thus reduce the inflammatory processes characteristic of advanced hypertension, coronary artery disease and heart failure.

Although the scavenging properties of polyphenol compounds have been extensively studied, there is insufficient evidence that these compounds can significantly reduce the impact of cardiovascular disorders like hypertension, coronary artery disease and heart failure once these have been established (Halliwell, 2007). The low bioavailability and high metabolic rate of these compounds could in part explain the absence of such clinical evidence (Hu, 2007). However, although they represent an important natural source that could help to prevent such disorders, at least in some instances they may act as a therapeutic alternative from a natural source

(Davison *et al*., 2010). *C. schiedeanus* would be one of these alternatives.

Isolated aortic rings represent a useful model to study potential vasodilator compounds. The aorta is not a resistance vessel, like the mesenteric artery, for instance, but a conductance vessel, and it is established that hypertension is a disorder due to an increase in resistance vessel tone. However, it is clear that several groups of antihypertensive drugs are active in this model, including: inhibitors of angiotensin converting enzyme (Arnal *et al*., 1994), angiotensin receptor blocking drugs (Zhang *et al*., 1995), beta blocking drugs (Lee *et al*, 1992) and calcium channel antagonists (Polster *et al*., 1990). Therefore, the vasodilation mechanism observed with *C. schiedeanus* in isolated aortic rings show the potential utility of this species when a reduction in vascular tone is needed, as in the case of hypertension.

CONCLUSION

In conclusion, *C. Shiedeanus* Schltd prevents experimental hypertension induced in rats with nitric oxide deficit, improving the endothelium vasodilatation response. These results give support to ethno botanic use of C. *schiedeanus* as a natural antihypertensive agent.

ACKNOWLEDGMENTS

This work was supported by the Universidad Nacional de Colombia, (VRI/DIB, grant numbers: 12785 and 15329). Thanks to the Animalarium in the Pharmacy Department of this institution. Thanks to Juliane HARTNACK for her English style corrections.

CONFLICTS OF INTEREST

There have been no conflicts of interest in carrying out this work.

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Received for publication on 15th April 2013
Accepted for publication on 31st July 2013