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Chemical Screening, Acute Toxicity and Analgesic Effect of the Aqueous Extracts of *Vitex madiensis* Oliv. (Lamiaceae-Viticoïdeae) and *Phytolacca dodecandra* L'Hérit. (Phytolaccaceae) Leaves

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Abstract: The purpose of this study is to identify the various secondary metabolites and evaluate the toxicity and analgesic activity of *Vitex madiensis* Oliv.*(VM)* and *Phytolacca dodecandra* L'Hérit.*(PD)* aqueous extracts two plants used in traditional medicine in Congo for the management of pain and other conditions. Phytochemical analysis ,by using tube reaction method, revealed very strong presence of saponins, strong presence of alkaloids and tannins from *PD;* strong presence of tannins, sterols and tri-terpenes from *VM*. Standard procedures using OECD Guideline helped to consider the *VM* extract non-toxic with an LD50 greater than 5000 mg / kg and the *PD* extract low toxic with an LD50 greater than 2000 g / kg in mice. Extracts promote an increase in body mass. Extracts tested at the doses of 50, 100 and 200 mg / kg inhibit significantly ($p < 0.001$) the abdominal cramps induced by acetic acid in mice. *PD* extract was more effective. Extracts decrease significantly ($P < 0.001$) the pain in the first phase (200 mg/kg) and second phase (50, 100 and 200 mg/kg) on formalin test.The signs of toxicity with *PD* extract and the inhibition of pain by both extracts would be due to the secondary metabolites presents in these extracts. The results obtained suggest that the analgesic effect of *VM* and *PD* may be mediated via both central and peripheral mechanisms.

Keywords: P. dodecandra, Vitex madiensis, Aqueous Extracts, Phytochemical, Toxicity, Analgesic

Introduction

Plants contain a molecular diversity of secondary metabolites (Rubin, 1988; Gurib-Fakim, 2008; Anton, 2010). This molecular diversity constitutes the interest of scientific research for the discovery of new active ingredients or the development of improved traditional medicines in order to combat pathologies. These strategies for the development of new drugs have the advantage of being beneficial to the environment, since they make it possible to limit the discharge of persistent organic pollutants caused by the synthesis of drugs by specific chemical processes (Mapongmetsem, 2006).

At present, many plants in Africa have been the subject of chemical and biological studies; in addition many herbal medicines are licensed in countries such as Mali, Senegal, Cameroon etc. (Pousset, 2006). However, only about 10% of the 400 000 plant species have been studied chemically and biologically (Quetin-Leclercq, 2002; Gurib-Fakim, 2008).

Many effective analgesics are available on the market, but the majority has significant adverse effects: morphine with emetic action and respiratory depression; Paracetamol can cause medullary aplasia and aspirin which can cause gastritis or

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thrombocytopenia (Moulin and Coquerel, 2002; Beaulieu and Lambert, 2010).

The objective of this work is to identify the various secondary metabolites and to evaluate the toxicity and analgesic activity of the aqueous extracts of the leaves of *Vitex madiensis* Oliv. (VM) and *Phytolacca dodecandra* L'Hérit. (PD) two plants used in traditional medicine in Congo (Bouquet, 1969; Adjanohoun, 1988; Sena Filho et al., 2008; Nzigidahera et al., 2009).

Material and methods

Plant material

Plant material is constituted by the leaves of two plants: *Vitex madiensis* Oliv. (Figure 1) and *Phytolacca dodecandra* L'Hérit(Figure 2). Both plants were collected in May 2015. The *leaves of VM* were collected in the south of Brazzaville (Congo), in the savanna. The leaves of *PD* were collected in a household of a district of Brazzaville (Congo). *VM* and *PD* were identified, respectively, by comparison with specimens NERE № 505 and J. TROCHAIN № 11270 of the national herbarium by Professor Jean-Marie MOUTSAMBOTE. These two samples were dried at room temperature and protected from the light, then pulverized, in the Laboratory of Animal Physiology and Experimental Physiopathology of the Faculty of Science and Technology of the Marien Ngouabi University at Brazzaville, in the Republic of Congo.

Figure 1: photography of the leaves of Vitex madiensis Oliv. Makana (Congo), in March 28th, 2015.

Figure 2: photography of leaves of Phytolacca dodecandra L' Hérit. Poto-Poto (Brazzaville-Congo), May 26th, 2016

Animal material

The animal material is constituted by females *Mus musculus* mice of Swiss albino strain weighing between 20 and 25 g, at least 3 months old. They were housed under standard conditions (25 ± 5° C, 40-70 % RH, 12h light/dark cycle) and fed with a standard died and water ad libitum. They were handled according to the standard protocols for the use of laboratory animals from OECD Guideline (OCDE, 2001).

Methods

Preparation of the extracts

The method of preparation used is decoction. It consists in boiling the leaves of each plant concerned (Elion Itou et al., 2014). After 30 minutes of boiling, the preparations are left to infuse a few minutes then filtered with some cotton. Decocts of both plants were then evaporated on a rotary evaporator at 50-60° C. The dry extracts obtained were used to prepare the various test solutions. 100 mg / ml of stock solution of, each plant leaves extract and paracetamol were prepared by dissolving 1000 mg in 10 ml of distilled water. 0.6% acetic acid solution was prepared by diluting 0.06 ml of 90% acetic acid in 10 ml of distilled water. The 5% formaldehyde solution was prepared by diluting 0.72 ml of 35% formaldehyde in 5 ml of distilled water.

Identification of secondary metabolites

The various secondary metabolites contained in the leaves of *VD* and *PD* L.Hérit. were determined by using the tube reaction method. classical phytochemical tests have been used for detection of alkaloids, tannins, anthocyanins, free flavonoids (flavones, flavanones, flavonols and flavanols), anthraquinones, sterols and tri-terpenes, saponosides, mucilages (Ibrahim et al., 2014).

Evaluation of acute toxicity

The method used to assess acute toxicity is those described in OECD Guideline No. 423 of of December 17th, 2001. The method consists in determining in which range of doses the substance is considered lethal. With a sequential process using three animals of the same sex per stage, the necessary information are obtained for the classification of the substance in a class of toxicity delimited by previously fixed LD50 values(Klansen Curtis et al., 2003). A determined dose of the substance is administered orally to a group of animals (Bounias, 1977). The absence or the presence of mortality related to the substance determines the next step, ie, the stop of the essay, the administration of the same dose to three additional animals, or the administration of a dose immediately superior or inferior to three additional animals. The animals were fasted for 4 hours before the administration of the extracts. Two

doses of each plant extract were successively used and administered orally, 2000 mg / kg then 5000 mg / kg. For every dose we used six animals, three per stage. Immediately after the administration of products, animals were observed individually at least once during the first four (4) hours and daily for 14 days. Observations concerned the modifications of the skin, the hairs, eyes, motor activity and behavior. Particular attention was put to the observation of the various manifestations of toxicity: tremor, convulsion, diarrhea, mortality and sleep. The individual weight of each animal was determined shortly before the administration of the test substance and then every two days until the end of the observation period. Weight changes were calculated and recorded.

Evaluation of analgesic activity

By acetic acid induced writhing assay

This method was described by Koster in 1959 and modified by Collier in 1968. It consisted in inducing a painful syndrome, using an injection of 0.6% acetic acid (0.1 ml / 10 g of weight)ip, in mice half hours (30 minutes) before the products administration. The painful syndrome is characterized by the stretching of the hind legs and twisting of the dorso-abdominal musculature. An analgesic would act by suppressing or reducing the manifestations of the painful syndrome. Mice were fasted for 24 hours and then divided into ten (10) lots of five (5) mice each. Both extracts were tested at doses of 50, 100 and 200 mg / kg per os. The distilled water was administered at the dose of 10 ml / kg. Paracetamol, a reference product, was administered also orally at doses of 50, 100 and 200 mg / kg. Stretchings and dorso-abdominal twistings characterizing the pain syndrome are counted for 10 minutes from the injection of acetic acid (Abena et al., 1997). The results were expressed in percentage of inhibition of stretching and dorso-abdominal twisting.

Formaldehyde test

This test was described by Debuisson and Dennis (1977). It consists in inducing pain in mice by intra-plantar injection of 10 µl of 5% formaldehyde. The painful syndrome characterized by the licking of the leg shows two phases: the first resulting from a direct stimulation of the nociceptors and the second corresponding to a sensitization phase involving the inflammatory phenomena. The intensity of pain is proportional to the time which the mouse puts to lick its paw. An analgesic would act by reducing this time or by deleting the lick of the paw. Animals were fasted during 24 hours, and then divided into 10 groups of three mice each. Both extracts were tested at doses of 50, 100 and 200 mg / kg. Distilled water was administered at 10 ml / kg and paracetamol at 50, 100 and 200 mg / kg. Except formaldehyde, all products were administered orally. The paw licking time characterizing the pain syndrome was taken during the first phase (0-5min) and the second phase (15-30min). The results were expressed in inhibition percentage of the paw licking.

Data processing

The statistical analysis was done using the Excel software (Office 2010) and the comparison of the means of the measures between lots was carried out by using the student's t test ($p < 0.05$; $p < 0.01$; $p < 0.001$).

Results

Chemical screening of aqueous extracts of the leaves of both plants

The various reactions in tube have revealed the different secondary metabolites contained in *VM* and *PD* extracts. The results are reported in Table I.

Table I: Secondary metabolites present in the aqueous extracts of leaves of *Vitex madiensis* Oliv. and *Phytolacca dodecandra* L'Hérit.

Secondary metabolites	***Vitex madiensis* Oliv.**	***Phytolacca dodecandra* L'Hérit**
Alkaloids	-	++
Tannins	++	++
Anthocyanins	+	-
Flavones	+	-
Flavanones	-	+
Flavanols	-	-
Free anthraquinones	-	-
Carotenoids	-	-
Sterols and tri-terpenes	++	+
Saponosides	+	++++++
Mucilages	++	++

+ Presence ; ++ = Strong presence ; ++++++= Very strong presence ; - = absence

Acute Toxicity of Aqueous Extracts of Leaves of Two Plants

Mortality

Table II show the results on the mortality recorded during the 14 days of the acute toxicity test. At the doses of 2000 and 5000 mg / kg, *VM* extract does not cause death. By contrast, for the *PD* extract, two deaths were recorded at a dose of 5000 mg / kg, but none at a dose of 2000 mg / kg. The first animal died 5 hours after the administration of the product, and the second 22 hours later.

Table II: Mortality recorded after oral administration of the aqueous extracts of the leaves of *Vitex madiensis* Oliv. and *Phytolacca dodecandra* L'Hérit. in mice.

Trial products and doses	Animal numbers	Mortality	Time of death
Distilled water (20ml / kg)	3	0	-
V. madiensis Oliv. (2000mg/kg)	6	0	-
V. madiensis Oliv. (5000mg/kg)	3	0	-
P. dodecandra L'Hérit. (2000mg/kg)	6	0	-
P. dodecandra L'Hérit. (5000mg/kg)	3	2	5th and 22nd Hour

Signs of toxicity observed

Tables III and IV show the observations made during the 14 days of the acute toxicity test. The mice which *VM* extract (2000 and 5000 mg / kg) and *PD* (2000 mg / kg), such as those given distilled water (20 ml / kg), showed a brief decrease in mobility after the administration of the product. Changes in hairs, skin, eyes, stool condition and mobility were not observed. By contrast, the mice which PD extract at the dose of 5000 mg / kg showed a loss of mobility, each mouse slept with its stomach against the cage in a corner. Of the 3 animals, two died, without recovering their mobility, at the 5^{th} and 22^{nd} hour after the administration of the extract. The surviving mouse returned to normal mobility 24 hours later.

Table III: Toxicity signs observed on mice which received *Vitex madiensis* Oliv. extract.

Parameters	Control group	Test group 1	Test group 2	Test group 3
Doses	20 ml/kg	2000 mg/kg	2000 mg/kg	5000 mg/kg
Behavior	N	N	N	N
Mobility	N	N	N	N
Condition of stools	C	C	C	C
Convulsion	A	A	A	A
Tremor	A	A	A	A
Sleep	A	A	A	A
Hair appearance	N	N	N	N
Aspect of the skin	N	N	N	N
Eye condition	N	N	N	N

N : normal, A : absence, C : compact, AN : abnormal, P : Loss of mobility (24h)

Table IV: Signs of toxicity observed in mice receiving the *P. dodecandra* L'Hérit**.** extract

Lots	Control	Test group 1	Test group 2	Test group 3
Parameters Dose	20 ml/kg	2000 mg/kg	2000 mg/kg	5000 mg/kg
Behavior	N	N	N	N
Mobility	N	N	N	P
Condition of stools	C	C	C	C
Convulsion	A	A	A	A
Tremor	A	A	A	A
Sleep	A	A	A	A
Hair appearance	N	N	N	N
Aspect of the skin	N	N	N	N
Eye condition	N	N	N	N

N : normal, A : absence, C : compact, AN : abnormal, P : Loss of mobility (24h)

Weight evolution

The weightings during the 14 days of the experiment helped follow the weight evolution of the treated and controlling mice. Figures 1 and 2 show the weight evolution of the mice. For the mice of the control group, there was no significant modification in weight. The figure 1 shows a significant increase ($p < 0.001$) in the weight of the mice on the 10^{th} day for group 1 and the 4^{th}, 7^{th}, 10^{th} and 13^{th} days for group 3. The mice of groups 1 and 2 were treated orally with a

dose of 2000 mg / kg and those of lot 3 a dose of 5000 mg / kg of aqueous extract of *VM.*

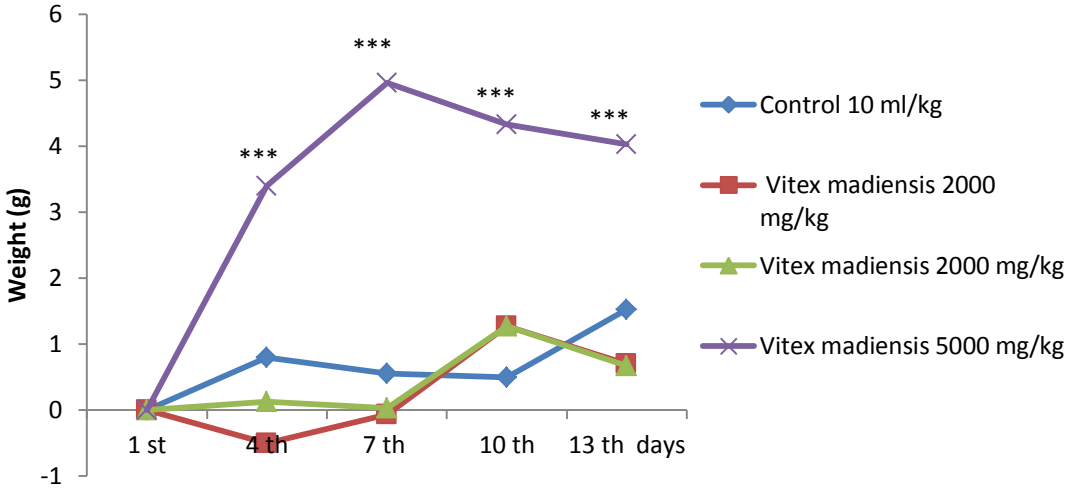

Figure 1: Evolution of the weight of mice treated with of V*itex. madiensis* Oliv. leaves aqueous extract . The values are averages ± ESM; n=3 for every lot. *** P < 0.001 compared to control lot.

Compared to the first day, Figure 2 shows a significant increase (p <0.001) in the weight at the 4th, 7th, 10th and 13th day for group 1; the 7th, 10th and 13th days for group 2. Mice of group 1 and 2 received orally a dose of 2000 mg / kg of *P. dodecandra* L'Hérit extract.

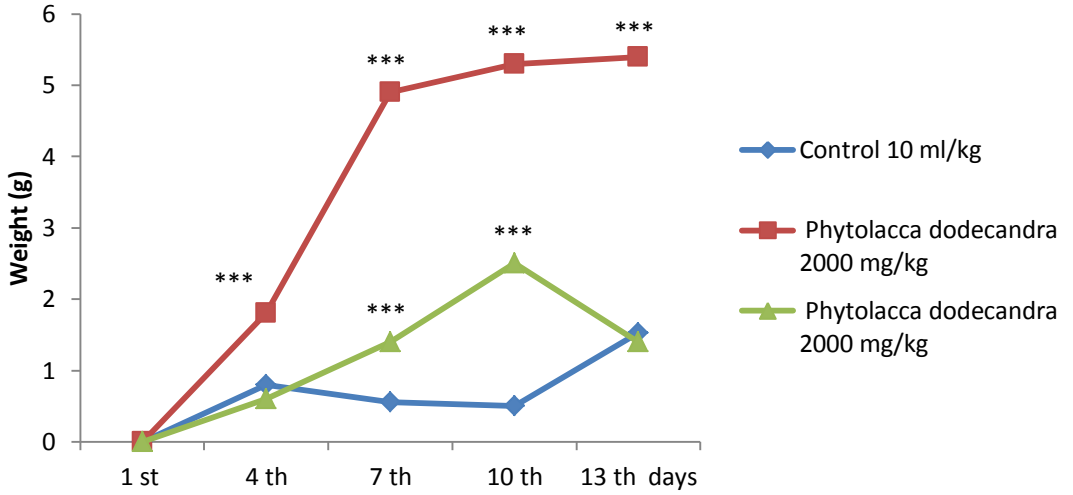

Figure 2: Evolution of the weight of the mice treated with the aqueous extract of the leaves of *P. dodecandra* L'Hérit. Values are means ± ESM; N = 3 for each group. *** p <0.001 compared to the control group.

Analgesic activity of aqueous extracts of leaves of two plants

Acetic acid test

Figure 3 shows the inhibitory effect of aqueous extracts of the leaves of *VM* and *PD* on pain syndrome induced by acetic acid. After injection of the acetic acid to the mice of the control lot, 53 ± 4.67 stretches and dorso-abdominal twists were counted. The *PD* extract reduces significantly (p <0.001) this number to 28.8 ± 1.35; 14.2 ± 1.83 and 5.2 ± 1.39 respectively at the doses of 50, 100 and 200 mg / kg. The *VD* extract also reduces significantly (p <0.001) this number to 24.4 ± 1.02; 21.4 ± 2.01 and 19 ± 1.87 respectively at the doses of 50, 100 and 200 mg / kg.

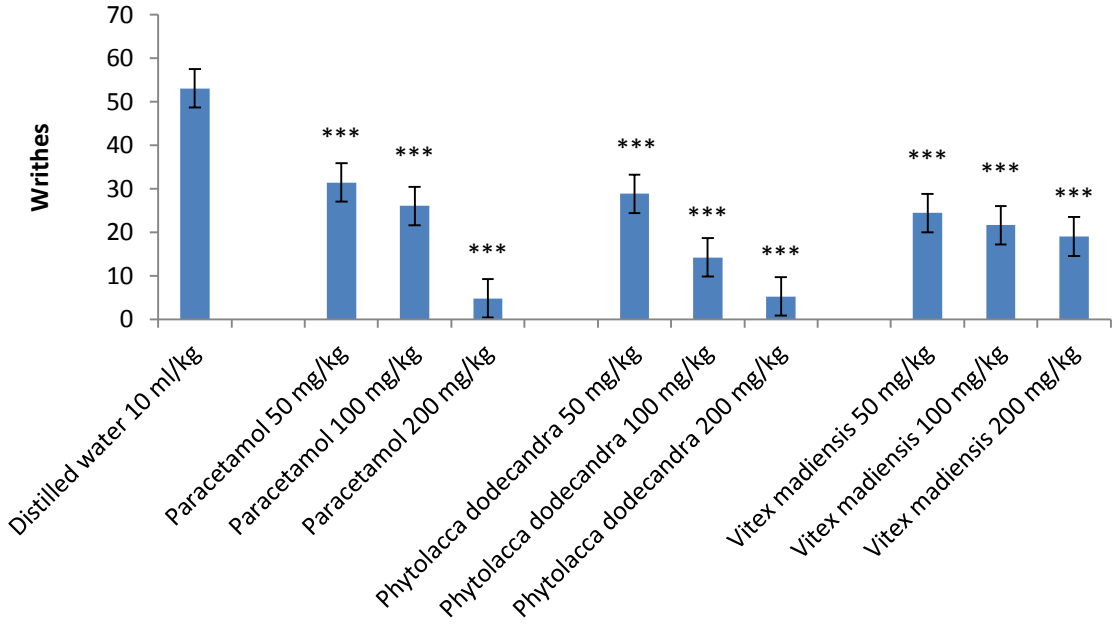

Figure 3: Antalgic effect of the aqueous extracts of the leaves of *Vitex madiensis* Oliv. and of *Phytolacca dodecandra* L'Hérit. on pain induced by intraperitoneal injection of acetic acid on mice. Values are means ± ESM; N = 5 for each lot. * P <0.05; ** p <0.01; *** p <0.001 compared to the control lot.

Formaldehyde test

Figure 4 and 5 show the effect of *VM* and *PD* extracts on the pain induced by injection of formaldehyde to mice. Time reaction is 41.27 ± 21.86 seconds for control group during the first phase (0-5 minutes). *PD* extract significantly decreased (p <0.001) this time to 12 ± 4.16 and 5 ± 4.04 seconds at the doses of 100 and 200 mg / kg, respectively. *VM* extract also reduced significantly (p <0.001) this time to 7.33 ± 4.05 s at a dose of 200 mg / kg.

During the second phase (15-30 minutes) time reaction was 113.04 ± 33.25 seconds for the control group. *PD* extract reduced significantly (p <0.001) this time to 27.46 ± 13.98 and 25 ± 5.50 seconds at the doses of 100 and 200 mg / kg, respectively. *VM* extract also reduced significantly (p <0.001) this time to 59.91 ± 24.48; 40.02 ± 11.37 and 22.08 ± 12.49 seconds at doses of 50, 100 and 200 mg / kg respectively.

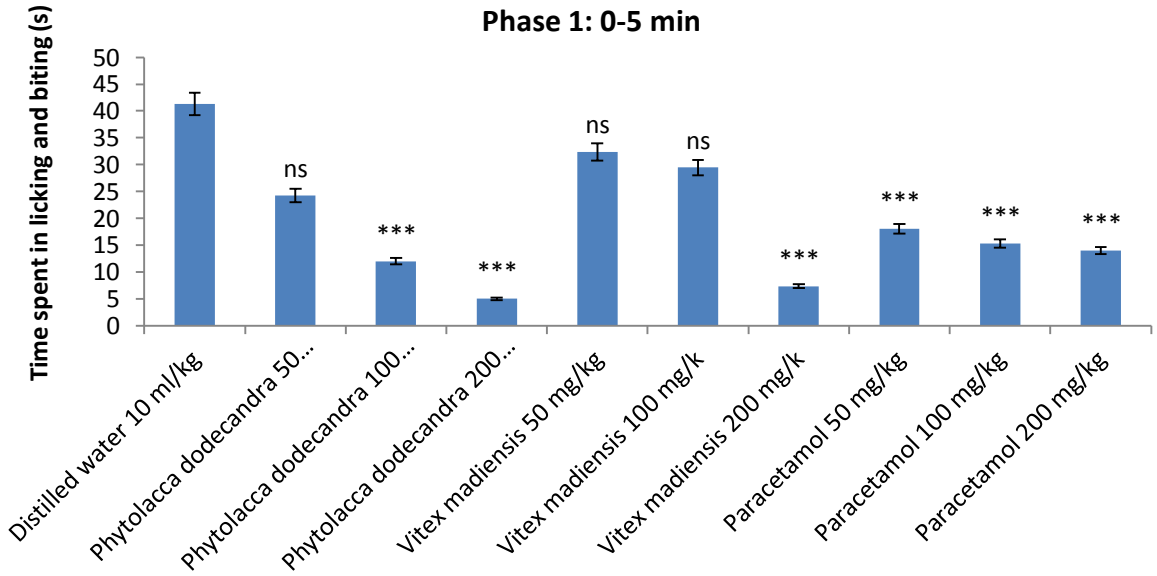

Figure 4: Analgesic effect of aqueous extracts of leaves of *Vitex madiensis* Oliv. and *Phytolacca dodecandra* L'Hérit. on pain induced by intra-plantar injection of formaldehyde in mice (phase 1: 0-5 minutes). Results

expressed as means ± ESM; N = 3 for each group. * P <0.05; ** p <0.01; *** p <0.001 relative to the control group, ns = not significant.

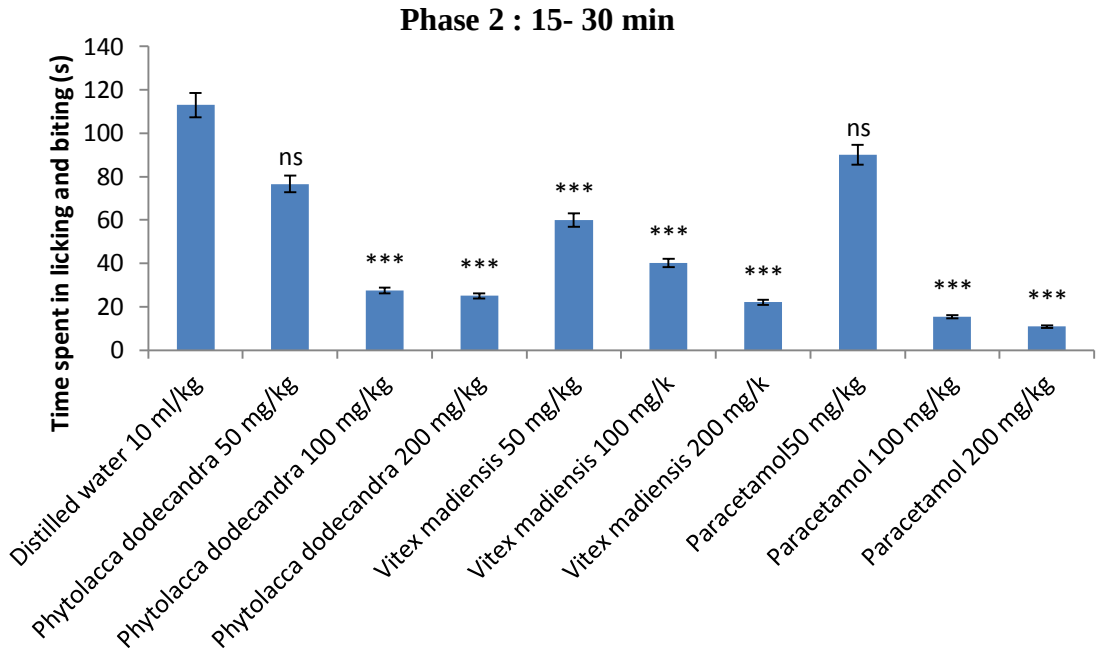

Figure 5: Analgesic effect of aqueous extracts of leaves of *Vitex madiensis* Oliv. and *Phytolacca dodecandra* L'Hérit. on pain induced by intra-plantar injection of formaldehyde in mice (phase 2: 15-30 minutes). Results expressed as means ± ESM; N = 3 for each group. * P <0.05; ** p <0.01; *** p <0.001 relative to the control group, ns = not significant.

Discussion

This study focused on the chemical screening and evaluation of analgesic activity and acute toxicity of aqueous extracts of the leaves of *VM* and *PD*, two food plants used in Republic of Congo. The presence of tannins, anthocyanins, flavones, steroids (Kubo et al., 1988), tri-terpenes, saponosides and mucilages has been demonstrated in the aqueous extract of *VM* (Kubo Isao et al.,1984). In *PD* aqueous extract, the presence of saponosides, tannins, flavanones, mucilages, alkaloids, steroids and tri-terpenes was demonstrated. Kibuyagi and Niyonzima (1978) had the same result with *PD* sample from Burundi (Niyongabo, 2003; Misganaw et al., 2012); and more recently Namulindwa et al.(2016) demonstrated the presence of these chemical groups with *PD* on the Uganda sample.

The acute toxicity test showed that up to 5000 mg / kg VM extract did not cause any mortality and sign of perceptible toxicity. Whereas, with *PD* extract two deaths were recorded at a dose of 5000 mg / kg within 24 hours of administration. The three mice that received the *PD* extract at 5000 mg / kg demonstrated immobility. The surviving mouse regained mobility 24 hours later. Paracelsus said: "Every substance is a poison and none is harmless, it is simply the dose that makes a substance harmless"(Davis, 1993). Based on the previously established LD50 classification (Viala, 1998), the *VM* extract is considered non-toxic with an LD50 greater than 5000mg / kg; and PD extract is considered low toxic with an LD50 greater than 2000 g / kg. Namulindwa et al. (2015) showed that up to the dose of 2048 mg / kg, PD extract did not cause any mortality in the Wistar rat, but made appear signs of toxicity such as decreased appetite, sleep and tremors. The death of the animals could be explained by the presence in the PD extract of alkaloids, saponosides and tannins, the toxic effects of which were reported (Jouzier, 2005; Mondoly and Poncelet, 2005 Pagin et al., 2011, Aubry, 2012). An in vitro toxicity study of VM *Vitex* extract on human cells showed no toxicity (Ondo et al., 2012).

Concerning weight gain, during the 14 days of the trial, we recorded for the extracts of *VM* at 5000 mg / kg and *PD* at 2000 mg / kg, a significant increase ($p < 0.001$) in body mass compared to the first day. Diet that exceeds energy requirements leads to an increase in body mass, insufficiency leads to weight loss (Grandjean et al, 1995; Murray et al, 2011). The control and treated lots of mice received the same amount of food, but no significant increase in body mass was observed for the control lot. The extracts of *VM* at 5000 mg / kg and *PD* at 2000 mg / kg would promote an increase in body mass. This effect could be explained by the presence of the steroids in these aqueous extracts. Duclos (2007) and Bigard (2008)

showed that steroids increased body mass (Kone et al., 2009; Nsonde Ntandou et al., 2015).

Both aqueous extracts tested at doses of 50, 100 and 200 mg / kg inhibited significantly ($p < 0.001$) the syndrome of pain-induced by intra-peritoneal injection of acetic. Acetic acid is a sensory irritant to the mouse (Bonnard et al., 2011). It induces pain by stimulation of phospholipases A2 which transforms the membrane phospholipids into arachidonic acid, the precursor of the synthesis of endogenous nociceptive mediators (Lazorthes, 1993, Collier et al., 1967, Guirimand, 2003 ; Boulanger and Beaulieu, 2010). Paul Ehrlich stated in 1909 that "substances do not act if they are not fixed" (Landry and Gies, 2008). The active molecules contained in *VM* and of *PD* would act by inhibition of the cyclooxygenases responsible for the synthesis of endogenous nociceptive mediators, such as prostaglandins (Lüllmann et al., 2010), from arachidonic acid. Among too plants, the extract of the leaves of *PD* is more effective. The PD extract showed a greater effect than paracetamol at the doses of 50 and 100 mg / kg and a neighboring effect at a dose of 200 mg / kg.

Formaldehyde induces pain in two phases: the first is due to the direct stimulation of nociceptors and the second involves inflammatory phenomena (Le Bars et al., 2001). *VM* extract inhibits significantly ($P < 0.001$), at the dose of 200 mg / kg a pain in the first phase, and at the doses of 50, 100 and 200 mg / kg the second phase which concern the inflammatory pain. *PD* extract inhibited significantly ($p < 0.001$) both types of pain at the doses of 100 and 200 mg / kg. Compared to paracetamol, the effect of *PD* extract is more important during the first phase. The inhibition of pain by aqueous extracts of *VM* and *PD* would be due to the presence in these extracts of chemical substances such as saponosides and alkaloids Rubin (1988) and Gurib-Fakim (2008) reported that these chemical groups possess analgesic properties(Nsonde Ntandou et al., 2010). At the end of this study, the efficacy and safety of these two plant extracts were shown. This study lays the foundation for analgesic therapy. Problems of extrapolation often arise because of the genetic differences that lead to different enzymes, therefore to a different metabolism of chemical substances, and effects such as psychic disorders and allergies that do not appear in animals, but since *VM* and *PD* are food plants, this extrapolation would be with no incident.

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