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Full Length Research Paper

Identification of a novel submergence response gene regulated by the *Sub1A* gene

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Submergence is one of the major constraints to rice production in many rice growing areas in the world. The *Sub1A* gene has been demonstrated to dramatically improve submergence tolerance in rice. Here, we report the identification of a novel submergence response (*RS1*) gene that is specifically induced in the *Sub1A*-mediated submergence tolerance response. Under submergence, *RS1* was upregulated in M202 (Sub1A) but downregulated in M202 in RNA-seq and microarray assays. Expression analyses of various tissues and developmental stages show that *RS1* mRNA levels are high in leaves and sheaths, but low in roots, stems, and panicles. Our results also show that *RS1* is highly expressed under submergence, drought, and NaCl stresses, but not under cold or dehydration stress. Hormone ABA treatment induces, whereas GA treatment decreases, *RS1* expression. The *RS1* and *Sub1A* genes are co-regulated under submergence. Overexpression of *RS1* in transgenic Kitaake (without *Sub1A*) and M202(Sub1A)×Kitaake do not result in enhanced submergence tolerance. Conversely, down-regulation of *RS1* in M202(Sub1A)×Kitaake lead to weaken submergence tolerance. We hypothesize that *RS1* may play a role in the *Sub1A*-mediated submergence tolerance pathway.

Key word: Rice (*Oryza sativa* L.), submergence, RNA-seq, *Sub1A*, abiotic stress.

INTRODUCTION

Submergence is one of main environmental stresses to rice growth and productivity in large rice-growing areas, especially in the flood-prone rainfed lowlands in South and Southeast Asia, where it regularly affects about 15 to 20 million hectares of rice land. Although rice is well adapted to aquatic environments, submergence causes annual losses of over US $1 billion in Asia (Xu et al., 2006). Fortunately, some indica cultivars, such as FR13A, can survive 10 to 14 days of complete submergence and renew growth after the floods recede. *Sub1A* was identified as the major gene conferring submergence tolerance in FR13A (Xu et al., 2006). *Sub1A*, an AP2/ERF transcription factor, was mapped on chromosome 9 and subsequently isolated from FR13A. Two alleles of *Sub1A*,

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Sub1A-1 and *Sub1A-2*, exist that differ only by a SNP variation at position 556. Allele *Sub1A-1* confers submergence tolerance while null allele *Sub1A-2* loses the function (Xu et al., 2006). *Sub1A* limits ethylene-promoted GA responsiveness during submergence by augmenting accumulation of the GA signaling repressors *SLR1* and *SLRL1* (Fukao and Bailey-Serres, 2008). *Sub1A* also enhances the expression levels of *PDC* and *ADH1*, thus providing the ATP needed for rice survival under submergence (Perata and Voesenek, 2007).

Studies on *Sub1A* have resulted in significant advancement in understanding submergence tolerance in rice. However, submergence tolerance is a complex trait orchestrated by many genes (Xu et al., 2006; Fukao and Bailey-Serres, 2008). The differences in submergence tolerance between different rice cultivars are only partly resolved since the identification of the *Sub1A* gene (Perata and Voesenek, 2007). For example, FR13A and Goda Heenati are two indica cultivars that carry the *Sub1A-1* gene. FR13A is a submergence tolerant cultivar, which can survive approximately two weeks under submergence whereas Goda Heenati does not (Xiong et al., 2012). The difference of submergence tolerance between FR13A and Goda Heenati indicate that other genes may interact with *Sub1A-1* that are necessary for *Sub1A-1* to confer submergence tolerance. Towards this goal, genes differentially expressed between FR13A and Goda Heenati under submergence, including two novel submergence responsive genes (Os09g0269900 and Os12g0202700), were identified using suppression subtractive hybridization and microarray approaches (Xiong et al., 2012). In addition, Seo et al. (2011) identified 24 proteins that interacted with *Sub1A-1* through yeast two-hybrid (Y2H) screens. However, the *Sub1A-1* gene is not sufficient for rice to tolerate more than two weeks under submergence (Septiningsih et al., 2012). Therefore, it is necessary to identify novel genes involved in *Sub1A*-mediated tolerance in order to investigate the molecular mechanism and further improve submergence tolerance.

RNA-seq technology has provided an effective way to obtain transcript sequences and abundance at the genomic scale, facilitating the identification of genes differentially expressed between different varieties (Grabherr et al., 2011; Trapnell et al., 2010). M202 (Sub1A) is an inbred rice line carrying the *Sub1A* gene, which leads to enhanced tolerance to submergence. Here, we performed RNA-seq to identify differentially expressed genes in M202(Sub1A) under submergence. We selected 38 increased genes and further tested their differential expression patterns under submergence both in M202 (a rice variety without *Sub1A* gene, and intolerance to submergence) and M202(Sub1A) by qRT-PCR. We identified a novel gene responsive to submergence, called *RS1*. The expression patterns of *RS1* in various tissues were also determined. The responses of *RS1* to various abiotic stresses, such as submergence, drought, cold, dehydration, and high NaCl, and to hormones, including ACC, GA, ABA, were assessed. However, overexpression of *RS*1 did not result in significant improvement of submergence tolerance in transgenic Kitaake (a rice inbred line without *Sub1A-1* gene). In M202(Sub1A)×Kitaake background, the seedling height was significant elongated with the down-regulation of *RS1*, indicating that the down-regulation of *RS1* resulted in weakened waterlogging tolerance. These results will help to explore the molecular mechanism of submergence tolerance in rice and facilitate breeding of submergence tolerant varieties.

MATERIALS AND METHODS

Plant growth and submergence treatment

Dehusked seeds were treated with 70% ethanol for 1 min, soaked in 1% sodium hypochlorite for 15 min, and rinsed with sterile water 4 to 5 times. Sterilized seeds were transferred to MS medium and grown in a chamber at 28°C for one week. Six one-week-old seedlings were planted at a silica sand pot (18 cm×16 cm), and were grown in the greenhouse at 25 to 28°C in a 16 h/8 h light-dark cycle. Three-week-old M202(Sub1A) seedlings were subjected to submergence for 0 or 3 days. Approximately 0.5 g leaves from six seedlings were detached. The leaf samples were ground to a fine power in the presence of liquid nitrogen. Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). All the experiments were performed with three biological replications. In total, 6 RNA samples were used for RNA-seq at BGI.

In order to accurately measure the increased seedling height under submergence, six sterilized seeds were planted into the tube (30 cm×2 cm). Six tubes each genotype were selected, and grown in the growth chamber at 25 to 28°C in a 16 h/ 8 h light-dark cycles. All experiments were performed with three biological replications. Seedlings of approximately 3 to 4 cm height were subjected to submergence treatment. Seedling heights were recorded every 24 h during submergence.

Identification of *RS1*

Under submergence, the identification of differentially expressed genes in M202 (Sub1A) was conducted according to Reiner et al. (2002). In previous research, our groups conducted microarray assays to identify the differentially expressed genes between M202 and M202 (Sub1A) under submergence (Jung et al., 2010). We combined the RNA-seq and microarray results and selected 38 differentially expressed genes that were upregulated in M202 (Sub1A) compared to M202 upon submergence treatment. The expression levels of these 38 genes under submergence were determined by qRT-PCR for both M202 and M202(Sub1A). Under submergence, the expression level of *RS1* was increased in M202 (Sub1A) but decreased in M202. Therefore, *RS1* was chosen as a candidate gene responsive to submergence.

Various environmental stresses and hormone treatment

All submergence, drought and hormone treatments were conducted with at least 3 independent biological replications. For gibberellin (GA), abscisic acid (ABA) and 1-aminocyclopropane-1-carboxylic acid (ACC) treatments, M202 and M202 (Sub1A) seedlings of 14 days old grown in germination paper were transferred into solutions

containing mock (0.1% DMSO), GA (10 μM), ABA (10 μM), and ACC (10 μM), respectively, and incubated for 6 h. Submergence treatment was carried out as described by Fukao and Bailey-Serres (2008). M202 and M202 (Sub1A) of 35 days old were subject to 0 or 5 days of drought stress in greenhouse. Seedlings of 14 days old were treated with stresses including dehydration (the intact seedlings were exposed in the air without water supply), salinity stress (using 150 mM NaCl solution), and cold exposure at 4°C (Xiong et al., 2014).

RNA isolation and expression analysis

All experiments were performed with three biological replications. Approximately 0.2 g tissues selected from six seedlings were used for RNA isolation. Leaves were selected from various treatments, including submergence, drought, cold, dehydration, NaCl, GA, ACC, and ABA. In addition, various tissues, including leaves, roots, shoots, stems, and panicles at different growth stages were also collected. First strand cDNA was synthesized using SuperScript-II reverse transcriptase according to the manufacturer's instructions (Invitrogen). The *actin 1* gene (Os03g50890.1) was used as an endogenous control to normalize expression data (Supplement Table 1). The qRT-PCR primers specific for the *RS1* and *Sub1A* genes are listed on Supplementary Table 1. Real-time PCR was conducted using the SYBR real-time PCR kit (Takara Japan) with IQTM SYBR® Green Supermixture according to the manufacturer's instructions (Bio-Rad USA). The reaction conditions were as follows: 94°C for 1 min; followed by 40 cycles of 95°C for 10 s, and then 55°C for 10 s.

Binary construct and rice transformation

The full coding sequence (CDS) of the *RS1* gene (Os02g0134200) encoding 255 amino acids (aa) derived from M202(Sub1A) was isolated by RT-PCR and transferred into the pCAMBIA1300 binary vector under control of the ubiquitin (Ubi-1) promoter to generate the *RS1* overexpression construct (*RS1ox*). The *RS1* RNAi construct (part sequence of *RS1* cDNA) under control of the CaMV35s promoter was transferred into the pBWA(V)HS vector by Wuhan Biorun Biotechnology company. The *RS1ox* and *RS1* RNAi constructs were transformed into Kitaake (a rice inbred line), respectively. Partial maps of the constructs including the loop sequence and the *RS1* sequence in the RNAi construct are shown in Supplementary Figure 1. Rice transformation was also conducted by Wuhan Biorun Biotechnology Company.

RESULTS

Differentially expressed genes specific to *Sub1A*-mediated submergence tolerance were identified

Differentially expressed genes were identified through comparing their expression levels based on RPKM (reads per kilobase of exon model per million mapped reads). The transcriptomic profiles of M202 (Sub1A) were determined by comparing their gene expression levels under normal condition and submergence treatment. Under submergence, 3712 genes were differentially expressed in M202 (Sub1A); 1696 of the 3712 genes were decreased and 2016 genes were increased (Figure 1). Our previous study using microarray has identified genes differentially expressed between M202 and M202 (Sub1A) under submergence (Jung et al., 2010). Here, we combined the RNA-seq and microarray results and selected 38 genes that increased in M202(Sub1A). Subsequently, their expression levels both in M202 (Sub1A) and M202 under submergence were determined by qRT-PCR (data no shown). Of particular interest, the expression level of *RS1* increased in M202 (Sub1A) but decreased in M202 (Figure 2) upon submergence. Under 1, 3, and 6 days submergence, the expression level of *RS1* in M202 (Sub1A) is approximate 2, 6, and 5 folds compared with that in M202 separately (Figure 2). Therefore, we chose *RS1* as a candidate gene for possible involvement in *Sub1A*-mediated submergence tolerance.

The *RS1* gene responds to drought and high salt stresses

We assessed changes of *RS1* expression levels by qRT-PCR when subject to various abiotic stresses, including drought, cold, dehydration, and high NaCl. The results show that the *RS1* expression levels both in M202 and M202(Sub1A) increased upon drought treatment, peaking at day 7 (Figure 3a). Under cold and dehydration stresses, the *RS1* expression levels remained unchanged (Figure 3b), suggesting that *RS1* does not respond to cold or dehydration stress. Under high concentration of NaCl, the *RS1* levels were significantly higher than those of untreated controls (Figure 3b). Therefore, the *RS1* gene responds to drought and high salt stresses.

RS1 is responsive to ACC and ABA treatments

Hormones play important roles in plant responses to abiotic stresses. Thus, we treated M202 and M202 (Sub1A) seedlings of 14 days old with ABA, GA, and ACC (a precursor of ethylene). The *RS1* expression levels were also determined by qRT-PCR. The results show that the *RS1* levels were significantly increased both in M202 and M202 (Sub1A) upon ACC treatment compared to untreated controls (Figure 4a). Under GA treatment, the *RS1* levels were increased both in M202 and M202 (Sub1A), but not statistically significant (Figure 4b). ABA treatment significantly increased the *RS1* levels both in M202 and M202 (Sub1A) (Figure 4c). In summary, the results show that ACC and ABA, but not GA, treatments induce *RS1* expression, indicating that the *RS1* gene is responsive to ACC and ABA hormones.

RS1 is mainly expressed in leaves and sheaths

The *RS1* expression levels in various tissues were determined using qRT-PCR. The transcript levels of *RS1* were low in shoots and roots of one-week and two-week old seedlings both in M202 and M202 (Sub1A). In one-

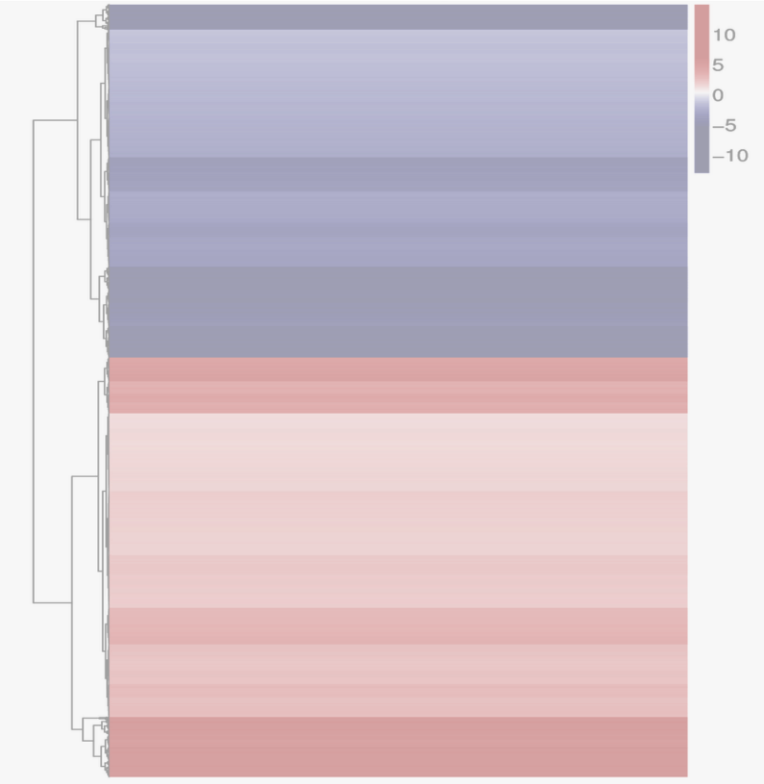

Figure 1. The clustering analysis of differentially expressed genes in M202(Sub1A) in response to submergence. Heat map of Pearsons correlation across 3712 genes differentially expressed under submergence for 2 days. The dendrogram of correlation between genes was displayed at the left of the heatmap.

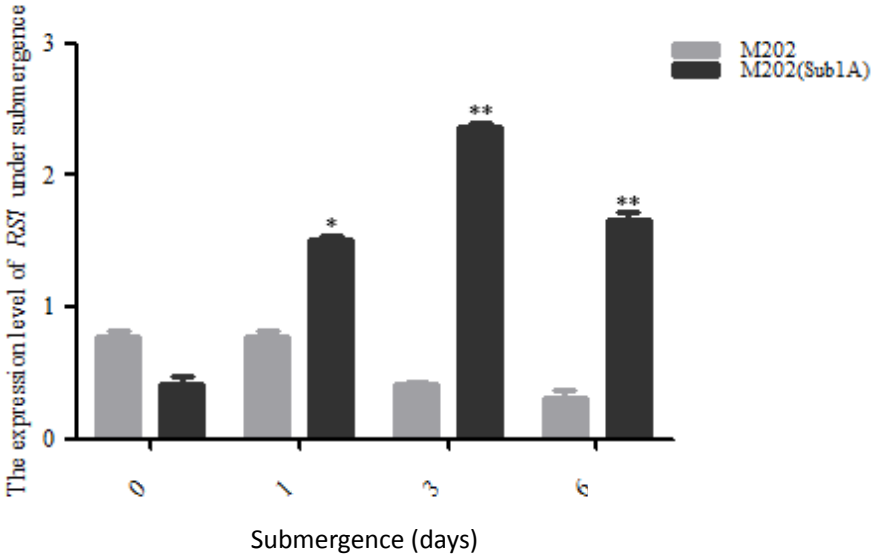

Figure 2. Expression levels of the *RS1* gene under submergence in M202 and M202(Sub1A). M202 and M202(Sub1A) seedlings of fourteen days old were subject to submergence treatment for 0, 1 3, and 6 days. The leaves were chosen for total RNA extraction and qRT-PCR experiments. The expression level of *RS1* was calculated using the 2^ddCt value. Each data point represents the mean±SD of 3 independent biological replicates.

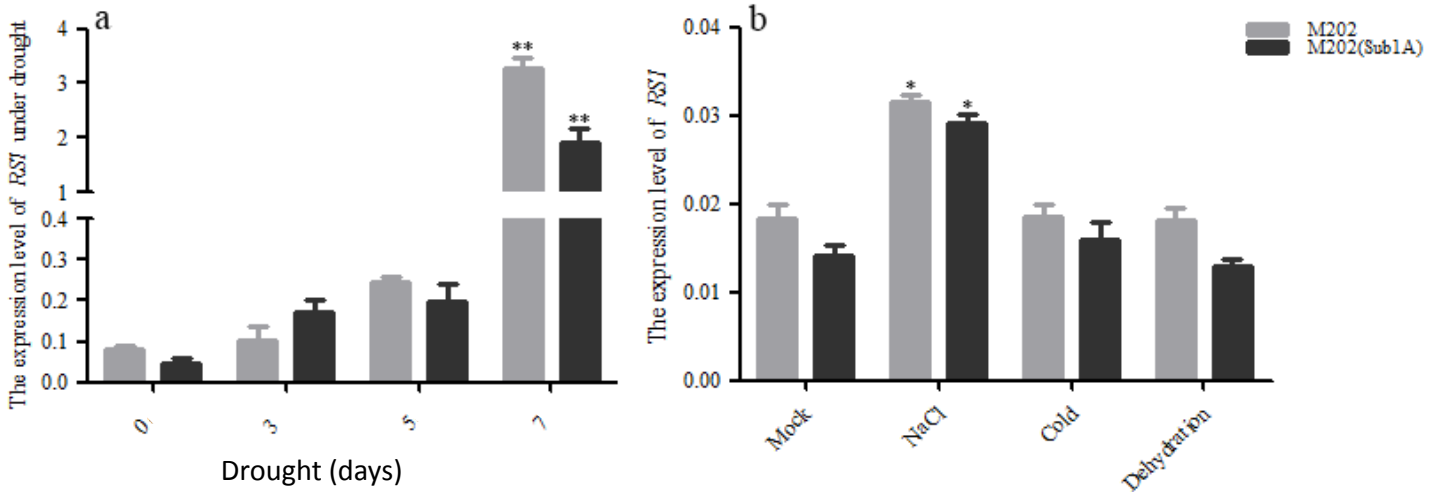

Figure 3. Expression levels of the *RS1* gene under drought, high NaCl, dehydration, and cold treatments. a. The *RS1* expression level under drought; b. The *RS1* expression levels under treatments with high NaCl, dehydration, and cold. All experiments were performed with three biological replications. Plants of 35 days old were subject to drought treatment. Fourteen-day old seedlings were treated with high NaCl, dehydration, or cold. Approximately 0.2 g leaves selected from six plants were used for RNA isolation. The expression levels of *RS1* under various stresses were determined by qRT-PCR. Each data point represents the mean±SD of 3 independent biological replicates. The student's t-test was performed. * indicates P<0.05; ** indicates P<0.01.

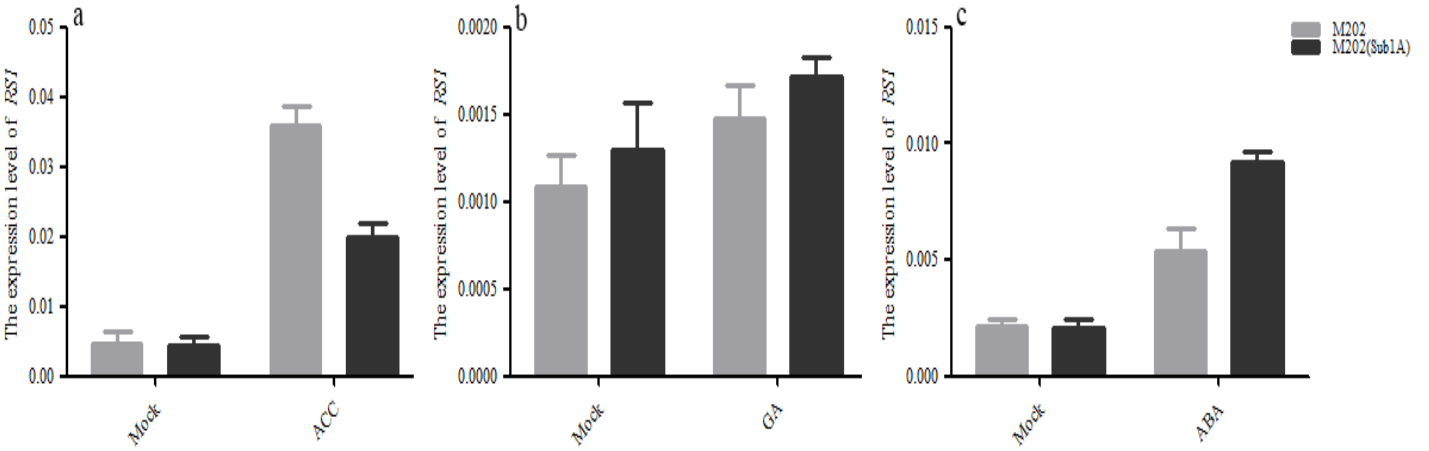

Figure 4. Expression levels of *RS1* under GA, ABA, and ACC treatments. Fourteen-day old seedlings were treated with GA (10 µM), ABA (10 µM), or ACC (10 µM) solution for 6 h. a. Expression level of *RS1* under ACC treatment; b. Expression level of *RS1* under GA treatment; c. Expression level of *RS1* under ABA treatment. Three biological replications were carried out. Approximate 0.2 g leaves collected from six seedlings each genotype was used for RNA extraction and qRT-PCR. The expression level of *RS1* was calculated using the 2^ddCt value. Each data point represents the mean±SD of 3 independent biological replicates. The student's t-test was performed. * indicates P<0.05; ** indicates P<0.01.

month old plants, the *RS1* levels were high in leaves and sheaths, but remained low in roots. At the reproductive stage, the *RS1* levels were high in leaf tissues, including the flag leaf, leaves near bolting, and leaves at bolting. The *RS1* levels remained low in stems and panicles (Figure 5). These results show that *RS1* is mainly expressed at high levels in leaves and sheaths, while only expressed at low levels in shoot, root, stem, and panicle tissues.

Overexpression of *RS1* in Kitaake did not enhance submergence tolerance

Constructs designed to overexpress (*RS1ox*) or to knockdown (RNAi) the *RS1* gene have been transformed into Kitaake separately. Twenty transgenic *RS1ox* and 16 transgenic RS1 RNAi independent lines were obtained. The homozygous transgenic lines were chosen in the T_3 generation, and their *RS1* expression levels were

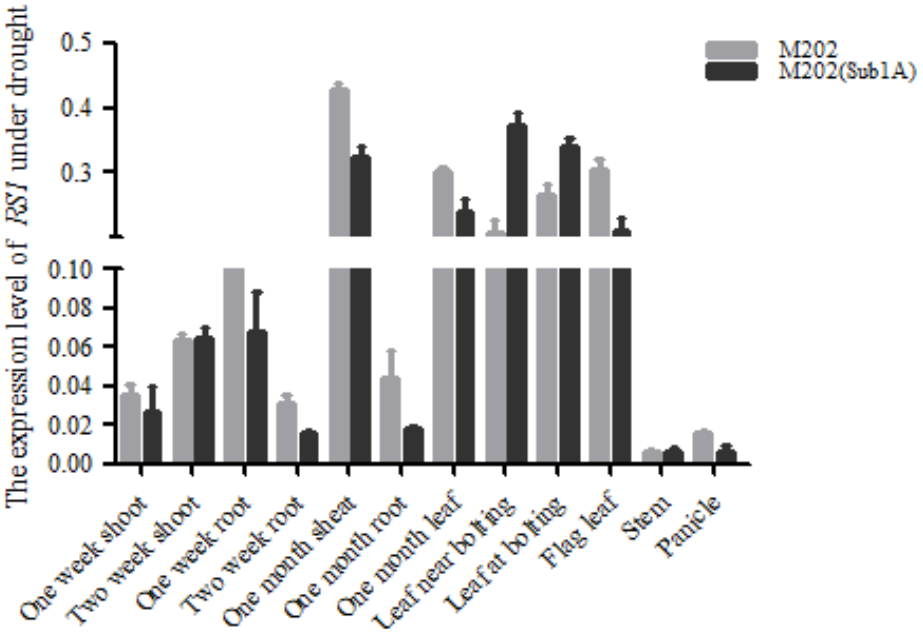

Figure 5. *RS1* expression levels in various tissues at different developmental stages. Approximately 0.2 g tissues were collected at various growth stages. The expression level of *RS1* was calculated using the 2^ddCt value. Each data point represents the mean±SD of 3 independent biological replicates. The student's t-test was performed. * indicates P<0.05; ** indicates P<0.01.

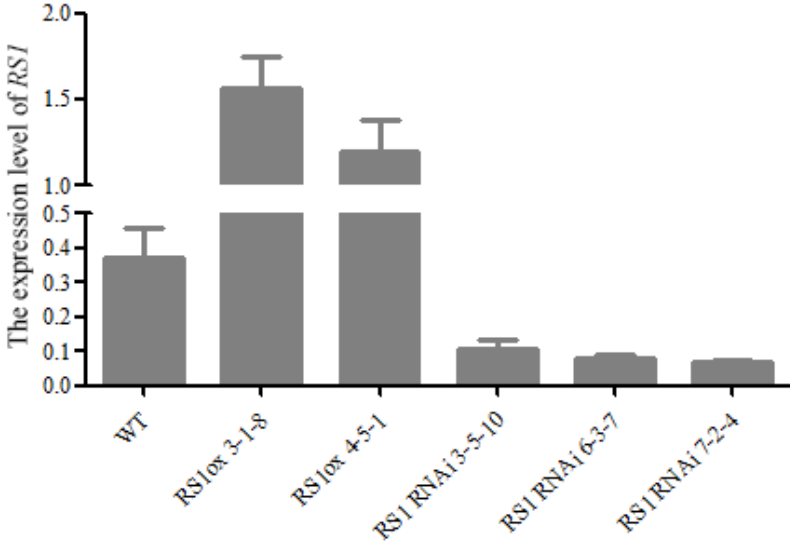

Figure 6. Expression levels of *RS1* in RS1ox and RS1 RNAi transgenic plants. For each transgenic line, approximately 0.2 g mature leaves collected from six plants were used for total RNA isolation. *RS1* expression levels were determined by qRT-PCR. Expression level was calculated using the rice *actin1* gene as the internal reference. Each bar represents the mean±SD of three independent biological replicates. Student's t-test was performed. * indicates p<0.05; ** indicates p<0.01.

measured by qRT-PCR. The results showed that the *RS1* expression levels in the transgenic *RS1ox* plants (# 3-1-8 and 4-5-1) were higher than others (Figure 6). Conversely, the *RS1* expression levels in the transgenic

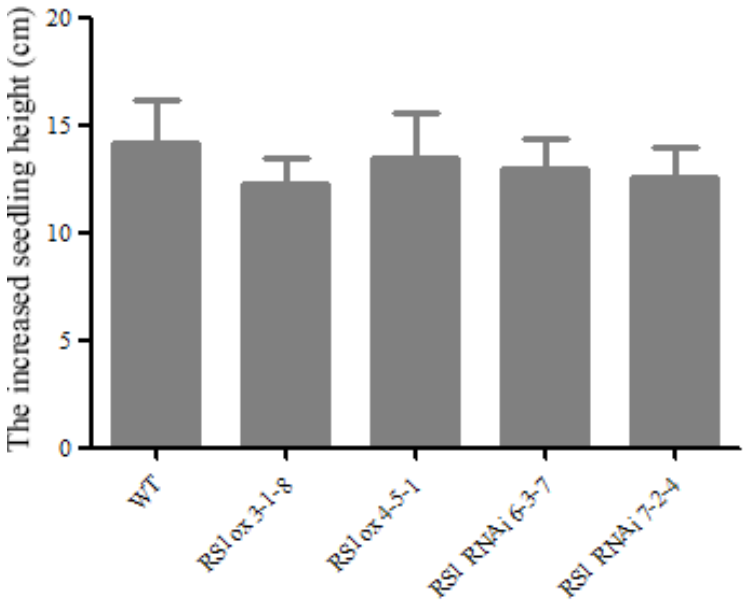

Figure 7. Increases in seedling height under submergence for 10 days. Seedlings of approximately 3 to4 cm grown in tubes were subject to submergence. The increases in seedling height were measured every 24 h. Each bar represents the mean±SE of three independent biological replicates. Student's t-test was performed. * indicates $p<0.05$; ** indicates $p<0.01$.

RS1 RNAi plants (# 3-5-10, 6-3-7, and 7-2-4) were lower than WT (Figure 7), indicating that the endogenous *RS1* was suppressed in the presence of the *RS1* RNAi construct.

Submergence-tolerant rice plants show stunted growth upon submergence and restart growth after the flood has receded (Xu et al., 2006). Thus, we subjected their seedlings planted in tubes to submergence tests, and measured their increased seedling heights. Under submergence for 10 days, transgenic *RS1ox* seedlings elongated (12.26 cm for line #3-1-8 and 13.47 cm for #4-1-5) slightly less than the WT (14.17 cm) (Figure 7), but not statistically significant. The similar results were also observed in transgenic *RS1* RNAi seedling (Figure 7). We did not observe other differences in submergence tolerance between *RS1ox* and WT plants, including seedling survival rates (data not shown). We conclude that the seedling elongation in transgenic *RS1ox* plants was not significantly different than that in WT upon submergence, indicating that *RS1ox* in rice did not result in significant improvement of submergence tolerance.

To measure the interaction between *Sub1A* and *RS1*, M202(Sub1A) was crossed with transgenic *RS1ox* and RNAi lines (Kitaake background), separately. The seedlings in F_1 generation were subjected to submergence. The increased seedling height was measured during submergence. With overexpression of *RS1*, the increased seedling height was unchanged compared with that of M202 (Sub1A)×Kitaake. Conversely, the seedling height was significant elongated with the down-regulation of *RS1* compared with the control even with the presence of *Sub1A* (Figure 8). Above results indicate that *RS1* may be play the role in *Sub1A* pathway to confer submergence tolerance in rice.

DISCUSSION

Reverse genetics approaches are widely used to identify genes of interest. In our previous and current studies, RNA-seq and microarray have been conducted to identify the candidate genes responsive to submergence in rice. *RS1*, a gene differentially expressed between M202 and M202(Sub1A) under submergence, was identified by both RNA-seq and microarray assays. M202(Sub1A), carrying the *Sub1A* gene, is a submergence tolerant line. Previous studies suggest that *Sub1A* limits ethylene-promoted GA responsiveness during submergence by augmenting accumulation of the GA signaling repressors *SLR1* and *SLRL1* (Perata and Voesenek. 2007; Fukao and Bailey-Serres, 2008). *ADH1* is a submergence tolerance marker gene, whose levels in M202(Sub1A) increased during submergence (Xu et al., 2006). Here we show that *RS1*, *Sub1A, ADH1, SLR1, and SLRL1* genes are co-induced upon submergence, reaching a peak within 24 h (Figure 9), indicating that *RS1* is tightly co-regulated under submergence with these well-characterized submergence-associated genes. The contents of GA and

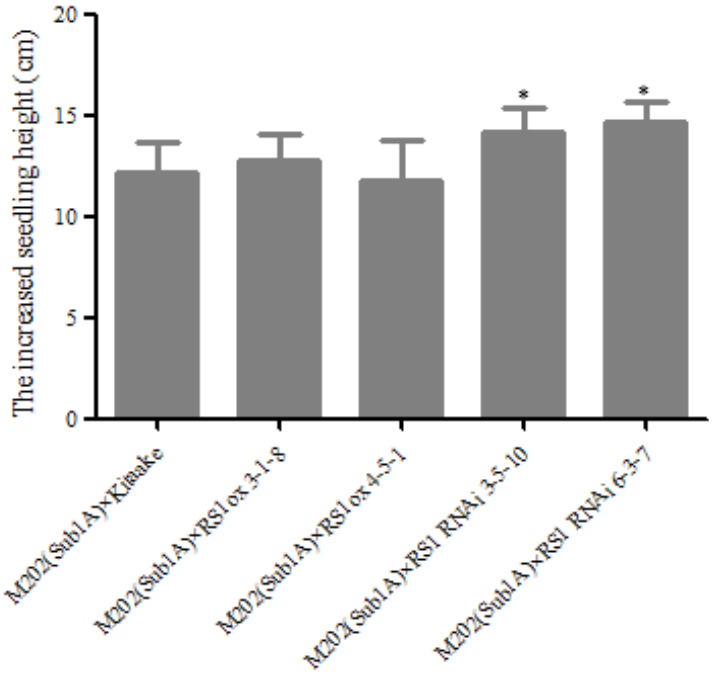

Figure 8. The increased seedling height in M202(Sub1A)×Kitaake background under submergence for 10 days. M202(Sub1A) was crossed with transgenic *RS1* or RNAi lines, respectively. In the F_1 generation, seedlings of approximately 3 to 4 cm were subjected to submergence. The increases in seedling height were measured every 24 h. Each bar represents the mean±SE of three independent biological replicates. Student's t-test was performed. * indicates $p<0.05$; ** indicates $p<0.01$.

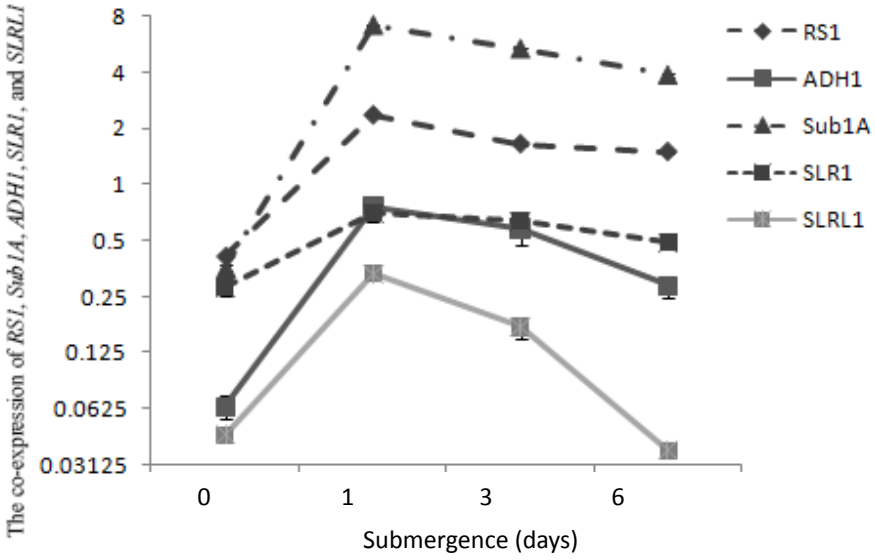

Figure 9. Co-expression of *RS1*, *Sub1A*, *ADH1*, *SLR1*, and *SLRL1* in M202(Sub1A) during submergence. Fourteen-day old seedlings were subjected to submergence for 0, 1, 3 and 6 days. All experiments were performed with three biological replications. The leaves from six seedlings collected from different time points during submergence were used for RNA isolation. Expression levels of *RS1*, *Sub1A*, *ADH1*, *SLR1*, and *SLRL1* were determined by qRT-PCR, and were calculated using the 2^ddCt value. Each data point represents the mean±SD of 3 independent biological replicates.

ABA play a role to suppress shoot elongation during submergence (Fukao and Bailey-Serres, 2008). The *RS1* expression level was changed by treatments with GA and ABA, indicating that *RS1* is regulated by GA and ABA. Together, these results suggest that *RS1* is associated with adaptation to submergence stress in rice.

Although *Agrobacterium*-mediated transformation technique has been widely applied to *japonica* rice, several *japonica* rice, including M202 (Sub1A), remain difficult to transformation (Roy et al., 2000). We subsequently used the Kitaake rice variety for *RS1* transformation because of its transformability and abundant genetic resources (Li et al., 2016). Although analysis of *RS1* suggests that *RS1* is responsive to submergence stress in M202 (Sub1A), overexpression of *RS1* did not result in significantly enhanced submergence tolerance in Kitaake. The different genetic backgrounds of M202 (Sub1A) and Kitaake, which does not contain *Sub1A*, may have contributed to these results. M202 (Sub1A), a near isogenic line of M202, possesses a 182 kb genomic fragment carrying the *Sub1A* gene derived from FR13A (Xu et al., 2006). When subject to submergence, the expression of *RS1* in M202 was greatly increased in the presence of *Sub1A*, but decreased in the absence of *Sub1A*. Therefore, we conclude that *RS1* positively responds to submergence stress regulated by *Sub1A*. To further pursue this issue, transgenic *RS1ox* and RNAi lines were crossed with M202 (Sub1A), respectively. With overexpression of *RS1* both in Kitaake or M202 (Sub1A)×Kittake, the increased seedling height do not significant decreased compared with their controls, indicating that overexpression of *RS1* do not result in enhanced submergence tolerance in rice. Conversely, with the presence of *Sub1A*, down-regulation of *RS1* in M202 (Sub1A)×Kittake lead to weaken submergence tolerance. Therefore, we conclude that *RS1* may be interacted with *Sub1A* to confer submergence tolerance in rice.

Conflicts of interests

The authors have not declared any conflict of interests.

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Abbreviations

RS1, Submergence response; **PDC,** pyruvate decarboxylase; **ABA,** abscisic acid; **GA,** gibberellin; **QTL,** quantitative trait locus; **ACC,**1-aminocyclopropane-1-carboxylic acid; **ADH,** Alcohol dehydrogenase; **SLR1,** Slender rice-1;**SLRL1,** SLR1 like-1; **ERF,** ethylene response factors; **RPKM,** reads per kilobase of exon model per million mapped reads; **qRT-PCR,** quantitative reverse transcription-PCR; **SNP,** single nucleotide polymorphism.

REFERENCES

Fukao T, Bailey-Serres J (2008). Submergence tolerance conferred by Sub1A is mediated by SLR1 and SLRL1 restriction of gibberellins responses in rice. PNAS 105(43):16814-16819.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng QD, Chen ZH, Mauceli E, Hacohen N, Gnirke A, Rhind N, Palma FD, Birren BW, Nusbaum C, Lindblad-Toh K, Friedman N, Regev A (2011). Full-length transcriptome assembly from RNA-seq data without a reference genome. Nat. Biotechnol. 29(7):644-652.

Jung KH, Seo YS, Walia H, Cao PJ, Fukao T, Canlas PE, Amonpant F, Bailey-Serres J, Ronald PC (2010). The submergence tolerance regulator *Sub1A* mediates stress-responsive expression of AP2/ERF transcription factors. Plant Physiol. 152:1674-1692.

Li GT, Chern M, Jain R, Martin JA, Schackwitz WS, Jiang L, Vega-Sánchez ME, Lipzen AM, Barry KW, Schmutz J, Ronald PC (2016). Genome-wide sequencing of 41 rice mutated lines reveals diverse mutations induced by fast-neutron irradiation. Mol. Plant. 9(7):1078-1081.

Perata P, Voesenek LACJ (2007). Submergence tolerance in rice requires Sub1A, an ethylene-response-factor-like gene. Trends Plant Sci. 12(2):43-46.

Reiner A, Yekutieli D, Benjamini Y (2003). Identifying differentially expressed genes using false discovery rate controlling procedures. Bioinformatics 19(3):368-375.

Roy M, Jain RK, Rohila JS, Wu R (2000). Production of agronomically superior transgenic rice plants using Agrobacterium transformation methods: present status and future perspectives. Curr. Sci. 79(10):954-960.

Seo YS, Chern MS, Bartley LE, Han M, Jung KH, Lee I, Walia H, Richter T, Xu X, Cao PJ, Bai W, Ramanan R, Amonpant F, Arul L, Canlas PE, Ruan R, Park CJ, Chen XW, Hwang S, Jeon JS, Ronald PC (2011) Towards establishment of a rice stress response interactome. PLoS Genet. 7(4):e1002020.

Septiningsih EM, Sanchez DL, Singh N, Sendon PMD, Pamplona AM, Heuer S, Machill DJ (2012). Identifying novel QTLs for submergence tolerance in rice cultivars IR72 and Madabaru. Theor. Appl. Genet. 124:867-874.

Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, Baren MJV, Salzberg SL, Wold BJ, Pachter L (2010). Transcript assembly and quantification by RNA-seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 28(5):511-515.

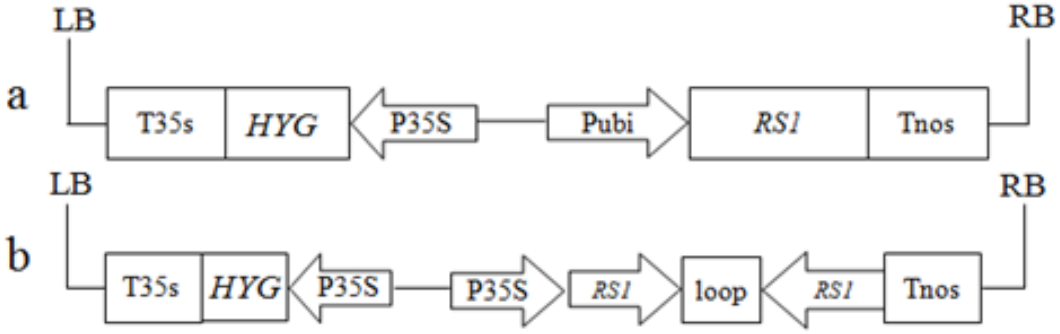
Xiong HY, Li JJ, Liu PL, Duan JZ, Zhao Y, Guo X, Li Y, Zhang HL, Ali J, Li ZC (2014). Overexpression of OsMYB48-1, a novel MYB-related transcription factor, enhances drought and salinity tolerance in rice. PLoS One 9(3):e92913.

Xiong HY, Yang J, Li YS (2012). Identification of submergence-responsive genes in two indica rice genotypes carrying SUB1A-1 but exhibiting differential tolerance. J. Plant Biol. 55:233-241.

Xu KN, Xu X, Fukao T, Canlas P, Maghirang-Rodriguez R, Heuer S, Ismail AM, Bailer-Serres J, Ronald PC, Machill DJ (2006). Sub1A is an ethylene-response-factor-like gene that confers submergence tolerance to rice. Nature 442:705-708.

Supplementary Table 1. Primers used in this study.

Primers	Sequence (5´-3´)	Usage
RS1 forward	CGCCGCCATCATCCAAACT	Real-time PCR
RS1 reverse	TCGGTGACCACGCCCTTCT	Real-time PCR
Actin1 forward	ACAGGTATTGTGTTGGACTCTGG	Real-time PCR
Actin1 reverse	AGTAACCACGCTCCGTCAGG	Real-time PCR
Sub1A forward	CGGCCTCATCACAATCGGAG	Real-time PCR
Sub1A reverse	ATGTCCATGTCCATATGTCGTCG	Real-time PCR
ADH1 forward	TGTTGGAGAGGGTGTGACTG	Real-time PCR
ADH1 reverse	GCCATCACCAATCATCACAC	Real-time PCR
SLR1 forward	GATCGTCACCGTGGTAGAGC	Real-time PCR
SLR1 reverse	GAGGGAATCGAACATGGTGG	Real-time PCR
SLRL1 forward	GGCGGCGACAATAACAACAACAGT	Real-time PCR
SLRL1 reverse	TACAAACACACGCTGCTACCATCC	Real-time PCR



Supplementary Figure 1. Schematic diagrams of *RS1* constructs. a The RS1ox construct in the pCAMBIA1300 vector (not to scale). b The *RS1* RNAi construct in the pBWA(V)HS vector. LB and RB, T-DNA left and right borders, respectively; Pubi, ubiquitin gene promoter; Tnos, nopaline synthase gene terminator; HYG, hygromycin resistance gene; T35s, CaMV 35S terminator; and P35s: CaMV35S promoter. The full-length cDNA of *RS1* was used in the *RS1ox* construct.

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Full Length Research Paper

Investigation of *Argania spinosa* L. (Skeels) polyphenols growing in arid and semi-arid conditions

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***Argania spinosa* L. Skeels, belonging to the *Argania* genus of the Sapotaceae family, is a species native to Morocco and Algeria. Due to its perfect adaptation to soil and climate, this tree plays an important ecological role in a constantly threatened encroached desert region. To understand the biological role of polyphenols in making the argan tree adapts to its natural habitat, we conducted a comparative study in two of the tree development stations: Tindouf located in South-western Algeria and Stidia located in Northwest Algeria. High performance liquid chromatography (HPLC) and Gas chromatography-mass spectrometry (GC-MS) used for the analysis of flavonoids led to the identification of six flavonols (two types of myricetin, rutin, hyperoside, quercetin, kaempferol) in the leaves of Tindouf argan tree, and two molecules (myricetin and quercetin) in the leaves of Stidia argan tree. Other molecules presented are few. The determination of flavonoids by spectrophotometry revealed the richness of Tindouf argan in these compounds (20%) compared to that of Stidia argan tree (8.7%). Histolocalisation of the flavonoids, tannins and anthraquinone in the leaves and stems of the tree was done using fluorescence microscope to understand the role of these molecules in the protection of this tree in its environment.**

Key words: *Aragania spinosa* L. (Skeels), histolocalisation, polyphenols, high performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS).

INTRODUCTION

Argania Spinosa L. (Sapotaceae) or commonly known as 'argan' is an endemic tree of Morocco and it grows also in the Southwestern Algeria, which is one of the most continental positions (Baumer and Zerraia, 1999). *A.*

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spinosa (L.) Skeels is the only species of the tropical family Sapotaceae growing in Nord Africa. Its oil extracted from the seeds is highly exploited by Morocco population. In addition to oil, the argan tree is traditionally used for other services such as wood for fuel (Charrouf and Guillaume, 1999). Also, the nutritional properties and culinary value of argan oil recently emerged from obscurity, and it has become the most expensive oil in the world (Lybbert et al., 2011).Today, argan oil of Morocco is employed in numerous cosmetic patents in fields in USA and Europe (Lybbert, 2007).

In Algeria, during the last two decades, the argan tree has been the subject of intensive investigation. We indicate the evaluation of the area of the argan tree in its natural habitat in Southwest Algeria estimated to 90.644 ha (Kechairi, 2009); the study of the seed structure and lipid composition (Errouane et al., 2014), the endocarp structure of the fruit to better understand its influence on germination capacity (Sebaa and Harche, 2014) and its role in the bio-sorption value of heavy metals (Hachem et al.; 2011 and Hachem et al., 2016).The argan tree was introduced in the Algerian coast (Stidia) (Baumer and Zerraia,1999).This tree involves a combination of morphological, physiological and metabolic mechanisms that reflect different types of adaptations. The purpose of this paper is to evaluate the polyphenols role in Argan relations in both development sites (Tindouf and Stidia). Stidia is located on the west coast of Northern Algeria. Tindouf is in the westernmost of southern Algeria. Indeed, these metabolites are widely distributed in plants either as soluble or cell wall bound compound as a result of interaction of a plant with its environment (Matern et al., 1995, Khallouki et al., 2005).

MATERIALS AND METHODS:

The healthy plant materials (leaves and stems) used for this investigation were collected in June 2011, from well-developed 30 trees in Tindouf location and 6 trees in Stidia location. Tindouf is located in South western Algeria at 27° 40N 8° 09W, 433 m altitude; it has an arid climate, its maximum temperature is 48°C from July to August, and its annual precipitation does not exceed 100 mm. Stidia site is located on the west coast of northern Algeria at 104 m altitude. It is characterized by a semi-arid climate, its maximum temperature is 25°C from July to August, and its annual precipitation is 524 mm. In this location, the number of argan trees is only 6.

Determination of the relative water content:

The relative water content is determined according to Hachem et al. (2011).

Qualitative analysis of flavonoids

Plant materials were dried and milled into uniform powders using a knife crusher of the type RETCH; they were placed in a mesh filter and then stored carefully until use. 100 mg plant materials were extracted with 10 ml Me OH 80%. After agitation (15 mn), the solvent is degassed in an ultrasonic bath (15mn) and then filtered (Millipore filter: 0.5 µm porosity). 30 µL of extract is injected for HPLC analysis.

Flavonoids are identified using their physicochemical properties: the comparison of their UV spectra with the spectra of known sample.

Standards

Methanolic solutions (200 µg mL^{-1}) of the following pure commercial substances were employed: Rutin, quercetin, myricetin, and kaempferol from Sigma. The purity of standard samples was assessed by HPLC (contents were greater than 98%). HPLC grade water was prepared by Milli-Q. All solvents were filtered through 0.5 µm (Millipore) membranes. Methanolic extracts were passed through C^{-18} Cartridge before use (Dohou et al., 2003). Liquid chromatography LC-MS mass spectrometry was used to confirm the analyses by HPLC

HPLC condition

HPLC separation was performed with an Ultimate 3000 Dionex system. The instrument was equipped with a RHEODYNE (100 µL) injector and a PAD UV/Vis detector. A NovaPak water filled with the same material was used. Two solvent mixtures were employed for elution A=water 0.1% formic acid and B=methanol. The solvent gradient consisted of linear increase in solvent B as follows: Initial conditions, 10%; 40 min, 60% and 43 min, 90%.

Separation was achieved at ambient temperature with a flow rate of 0.8 mL/min.

LC-MS condition

LC-MS analyses were performed with an Acquity UPLC coupled to a Xevo-G2Q-Tof (waters) mass spectrometer. Chromatographic separations were performed on a 100 mm × 2.1 mm × 1.7 µm Acquity UPLC BEH C 18 column (Water) maintained at 35°C. The binary solvent system included A=water +0.1% HCOOH and B=acetonitrile. The solvent gradient consisted of linear increase in solvent B as follows: Initial conditions, 10%; 12 min, 100%. Flow rate was 300 µL/min.

Phenolic compounds were detected using negative mode with the following settings: Capillary voltage, 2 KV; cone voltage, 30 V; source temperature, 130°C; desolvation temperature, 450°C. Nitrogen was used as both cone gas (20 L/h) and desolvation gas (750 L/h). Argon was used as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution. To ensure accuracy and reproducibility, analyses were acquired using the leucine-enkephalin in real-time as the lock mass at a concentration of 500 pg/µL and flow rate of 2 µL/min.

The samples for the assays and the identification of flavonoids were collected in June 2011. They were dried in an oven at a temperature of 45°C. and then powdered and stored from light until use.

Determination of total flavonoids

2 mL of alcoholic was mixed with 100 µL of Neu. Absorbance at 404 nm was determined and compared to quercetin standard (0.05 mg/mL) treated with the same amount of reagent. The percentage of total flavonoids is calculated by quercetin equivalent using the following formula (Lebreton et al., 1967):

F%=(0.05×A ext/Aq)×100/C ext

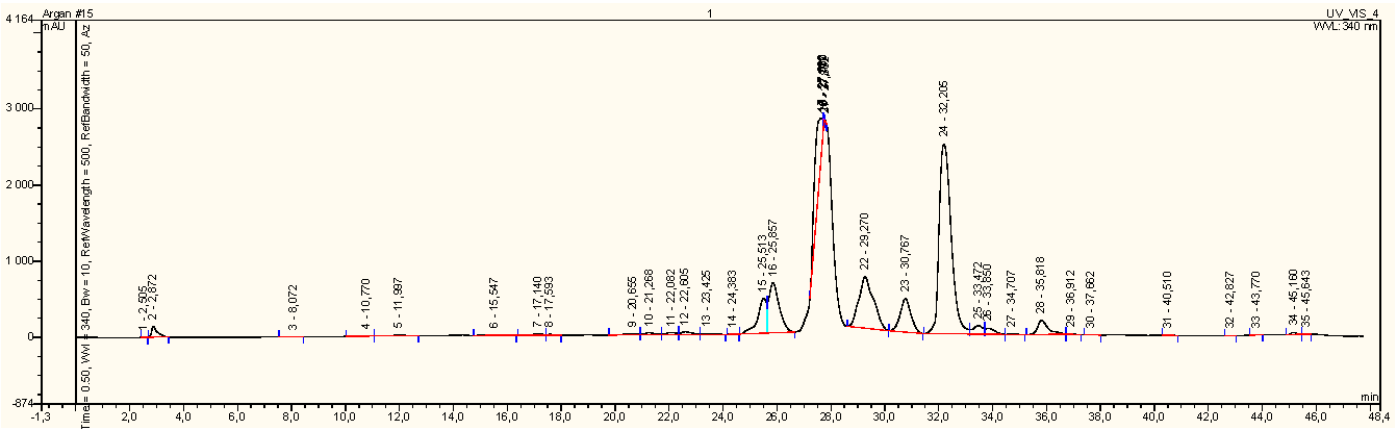

Figure 1. HPLC analysis of flavonoids of Tindouf argania leaves. The numbers corresponding to the different flavonoid (Stable 1).

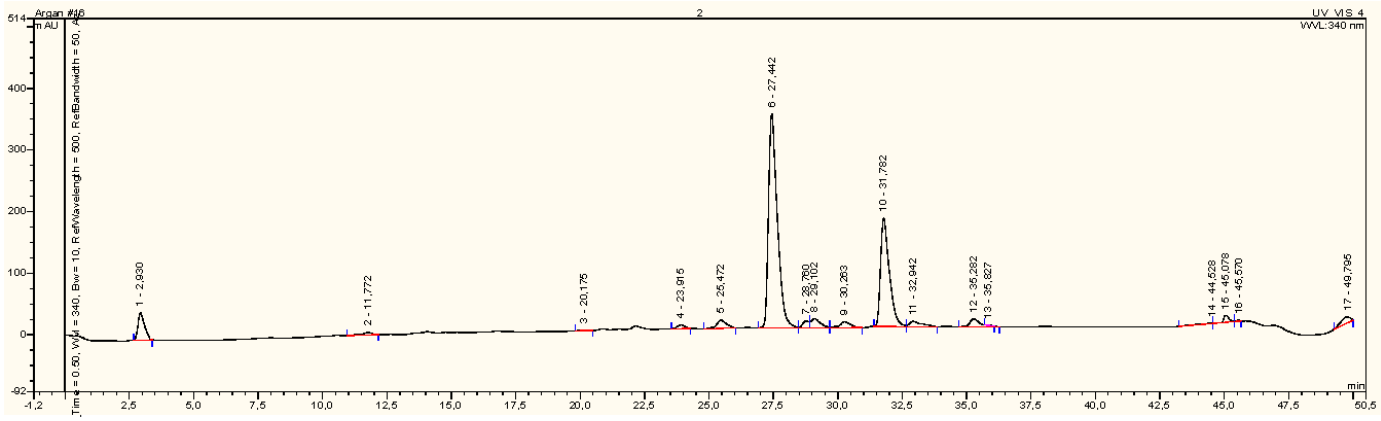

Figure 2. HPLC analysis of flavonoids of Stidia argania leaves.

A ext: Absorption of the extract; Aq: Absorption of the quercitin.

Histolocalisation of phenolic compounds studied on the leaves and stems section

Flavonoids

The leaf and stem sections are immersed in Neu reagent (1956) for 2 min and then placed between slide and cover slip. They were observed using a fluorescence microscope. The flavonoids had dull yellow fluorescent colour .

Tannins

Tannins are detected by vanillin sulfuric acid reagent: The sections were placed for 10 min in this reagent. The tannins are colored red.

Anthraquinones

The sections were placed for 10 min in a solution of 1% KOH and mounted between slide and cover slip. They were observed under light microscope. Anthraquinones color is orange.

RESULTS AND DISCUSSION

The water content in the leaves of Tindouf argan tree is greater than that in the leaves of Stidia (85% against 55% respectively). The relative water content of leaves of the argan tree is higher than the leaves of the argan tree growing in Tindouf, where aridity is strong. The water retention is probably due to the strategy used by the stomata to curb evapotranspiration. Indeed, similar observations were described by Epstein and Grant (1973) and El Aboudi (1990). The total flavonoids in fractions expressed as quercitin equivalents are 20% in the leaves of Tindouf argan tree; they are 8% in the leaves of Stidia. This difference observed between the two samples indicates that the phenolics content varies based on weather conditions. The Argan tree growing in Tindouf is located in extreme conditions of stress; it reacts by increasing its phenolic pool. These observations are similar to those of Domingo et al. (2003) on other species.

The observation of chromatographic profiles of the

Table 1. Qualitative analysis of flavonoids of the argan tree leaves.

Pic No. UV	Compounds	TR (min)	The absorption in the spectrum
1	Myricetine a	25.85	209, 258, 353
2	Myricetine b	27.80	209, 261, 351
3	Rutine	29.27	205, 256, 355
4	Hyperoside	30.76	206, 256, 353
5	Quercetine	32.20	207, 258, 350
6	Kaempferol	35.81	196, 266, 350

TR, Time retention.

extracts of the leaves by HPLC shows that the leaves of Tindouf argan tree have six flavonoid molecules (Myricetin a, myricetin b, rutin, hyperoside, quercetin, kaempferol) (Figure 1), while those of Stidia have two predominant molecules (myricetin b and quercetin) (Figure 2). Other molecules are present in trace (Table 1).

HPLC analysis confirms the existence of two flavonoids in the extracts of the leaves of Tindouf argan tree (quercetin, hyperoside) and their absence in the argan tree of Stidia. These biomolecules are characterized by their ability to absorb UV radiation in the wavelength range from 280 to 315nm (Reuber et al., 1996). This enables the adaptation of Tindouf argan tree to high altitude (530 m) where UV radiation is intense. The absence of these molecules in Stidia leaves suggests that UV radiation is less intense at altitude where Stidia is located. The wavelength of 310nm radiation penetrates less deeply into the leaves rich in glycosides of flavones (Ryan et al., 2002). This property of o- diphenols is related to their antioxidant properties besides the direct effect of their filter screen , a second level of action that traps and neutralizes the activated forms of the oxygen and free radicals formed by the action of UV -sensitive molecules (Macias et al., 2003).

In the the histolocalisation of leaf phenolics, due to their fluorescence property, their accumulation sites that are related to biotic and abiotic factors are shown. Flavonoids are present mainly in the upper epidermal seat (Figure 3). Chlorophyll, which appeared red in the presence of Neu reagent, can hide flavonoids present in the palisade parenchyma Chlorophyll.

Tannins (Figure 4) and anthraquinones (Figure 5a and b) are present in the palisade and spongy parenchyma.

In the stem, flavonoids are located in the epidermal cell and phloem. They are adsorbed in cell walls (Figure 6). The presence of these molecules in the outermost cells of the leaves and stems reduces UV penetration in the parenchyma.

A kinetic study of the radiation wavelength of 310 nm on barley leaf using optical fibers (Reuber et al., 1996) showed that the radiation penetrates less deeply into the leaves of a variety rich in flavonoids than in a poor mutant containing little flavonoids (Stapleton and Walbot, 2004).

These metabolites play the role of UV filter; protect

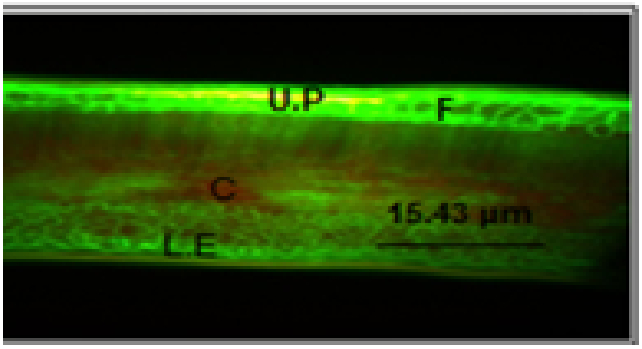

Figure 3 Location of flavonoid in leaves. C, cortex ; F, fluorescence; L.E, lower epidermis; UP, upper epidermis.

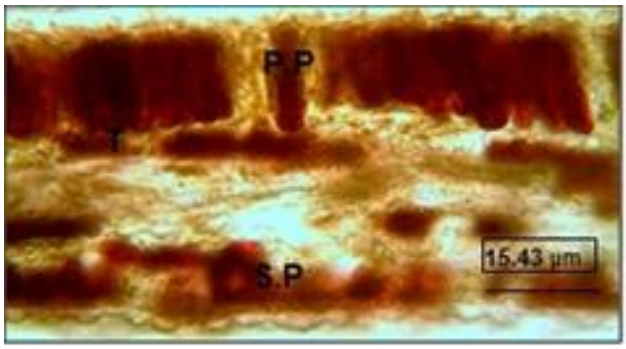

Figure 4. Location of tannins. SP, spongy parenchyma; P.P., palisadique parenhyma; T, tannin.

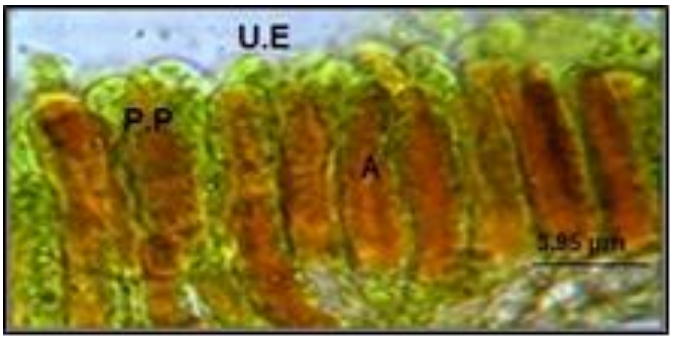

Figure 5a. Location of anthraquinones at the palisade parenchyma. U.E, upper parenchyma; PP, palissadique parenchyma ; A, anthraquinones.

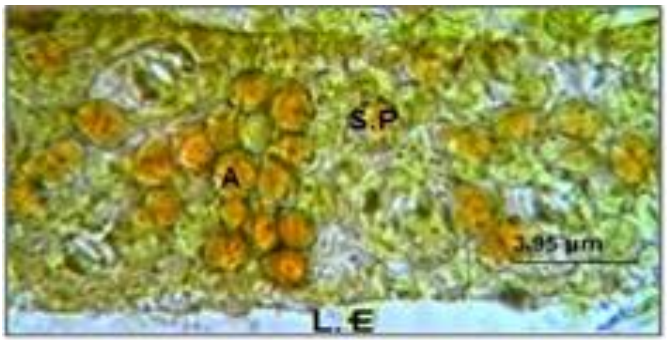

Figure 5b. Location of anthraquinones at the spongy parenchyma. A, anthraquinones; LE, lower epidermis; SP, spongy parenchyma.

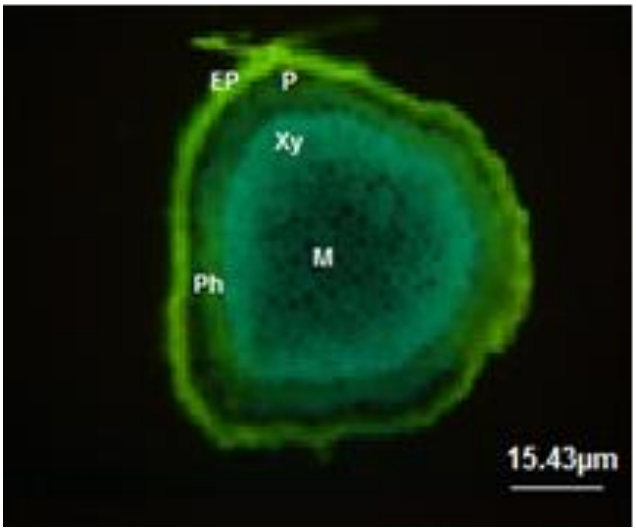

Figure 6. Location of flavonoids in stem.

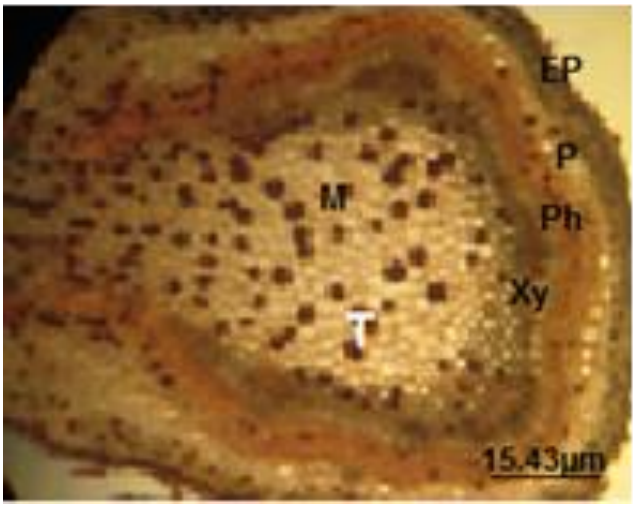

Figure 7. Location of tannins in stem. Ep, epidermis; M: pith; Ph, phloem; P, parenchyma; Xy, xylem.

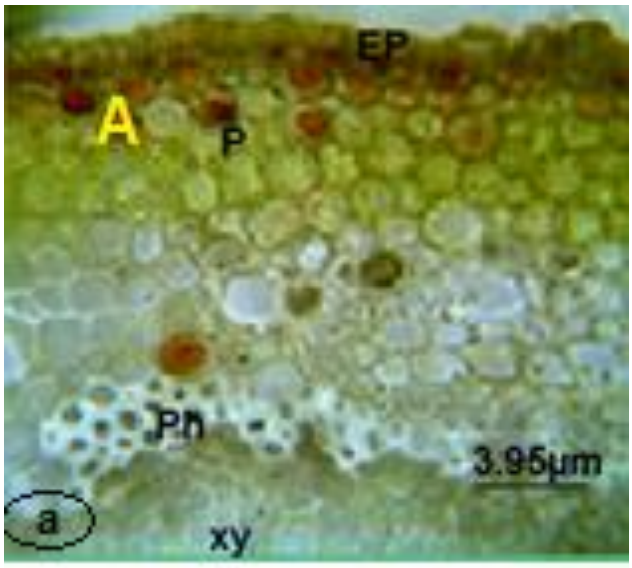

Figure 8a. Location of anthraquinones in the stem. A, anthraquinones; P, parenchyma; Ph, phloem; xy, xylem.

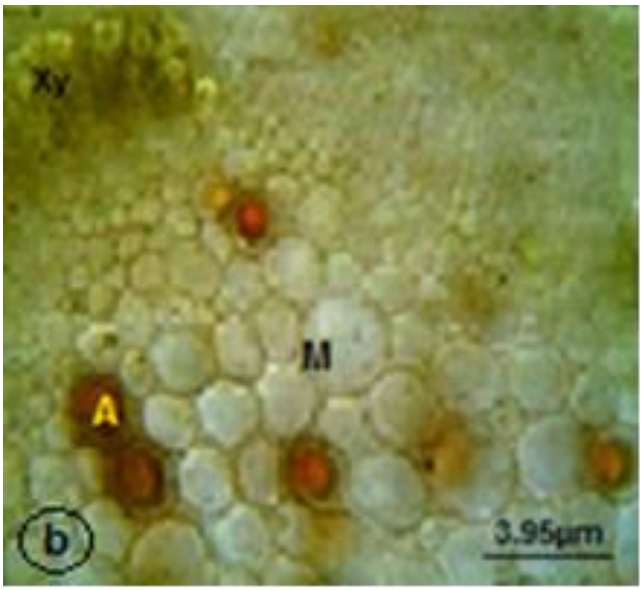

Figure 8b. Location of anthraquinones in the stem. A, anthraquinones; M, pith; Xy, xylem.

some macromolecules such as DNA, RNA, photosynthetic pigments, proteins and lipid membran (Moiseev et al., 2011). Tannins are observed in the cortical parenchyma, phloem and pith (Figure 7).

The tannins are present as sufficient hydrosolubility to be accumulated in the vacuole. The presence of these molecules in various tissues shows their importance for defense: It protects the tree against all attacks from animals or microorganisms due to their capacity to bond to proteins in solution and to precipitate them.

Anthraquinones is located in epidemic cells and

cortical parenchyma (Figures 8a and b). Anthraquinones are often present in the plant under heteroside (ose and aglycone), and it is only after hydrolysis that the quinone is free (Jignasu and Mehta, 2012). Quinone is an aromatic compound that bounds to isoprenique chain and has essential biological functions, in particular the transfer of electrons.

The peripheral location of flavonoids and tannins at the level of the sheets of the Argan tree constitutes a protective screen for the most internal tissues against UV radiations and other attacks of the external environment.

Conclusion

This preliminary study on the explanation of the role of secondary metabolites (flavonoids, tannins and anthraquinones) in making the argan tree adapts to its environment shows that the tree has a strategy to adapt to drought. This is due to its ability to retain water and synthesize secondary metabolites. Quercetin and myrecitine b are present in Tindouf argan leaves and Stidia argan leaves but myrecitine a, rutine, hyperoside are present only in Tindouf argan leaves. This difference in the composition of Tindouf argan tree located in arid zone and Stidia argan tree located in semi- arid zone indicates the adaptation of *A. spinosa* to its environment.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Baumer M, Zeraïa L (1999). La plus continentale des stations de l'arganier en Afrique du nord. Rev. For. Fr.3 : 446-452.

Dohou N, Yamni K, Tahrouch S, Idrissi Hassani LM, Badoc A, Gmira N (2003). Screening phytochimique d'une endémique ibéro- marocaine, *Thymeleae lythroides* . Bull. Soc. Pharm. Bordeaux 142:61-78.

Domingo F, Brenner AJ, Gutierrez L, Clark SC, Incoll LD, Aguilera C (2003).Water relations only partly explain the distributions of three perennial plant species in a semi-arid environment. Biol. Plant. 46(2):257-262.

El Aboudi A (1990). Typologie des agraneraies inframediterraneennes et écophysiologie de l'arganier (*Argania spinosa* (L.) Skeels dans le Sous (Maroc). Thèse de doctorat. Université Josep Fourier Grenoble I, 123 p.

Epstein E, Grant WJ (1973).Water stress relations of the potato plant under field conditions. Agron. J. 65:400-404.

Errouane Kh, Doulbeau S, Vaissayre V, Leblanc O, Collin M, Kaid-Harche M, Dussert S (2014). The embryo and the endosperm contribute equally to argan seed oil yield but confer distinct lipid features to argan oil. Food Chem. 181:270-276.

Hachem K, Astier C, Chaleix V, Faugeron C, Krausz P, Kaid -Harche M, Gloaguen V (2011).Optimization of lead and cadmium binding by oxidation of biosorbent polysaccharidic moieties. Water Air Pollut. 223:3877-3885.

Hachem K, Benabdesslem Y, Ghomari S, Hasnaoui O, Kaid-Harche M (2016). Partial structural characterization of pectin cell wall from *Argania spinosa* leaves. Heliyon 2(2):e00076.

Jignasu P, Mehta (2012). Separation and characterization of anthraquinone derivatives from *Cassia Fistula* using chromatographique and spectral techniques. Int. J. Chem. Sci. 10(1):306-331.

Kechairi R (2009). Contribution à l'étude écologique de l'Arganier Argania spinosa (L.) Skeels dans la région de Tindouf (Algérie). Thése de magister, Univ. Houari Boumediene. Alger 95 p.

Khallouki F, Spiegelhalder B, Bartsch H, Owen RW (2005). Secondary metabolites of the argan tree (Morocco) may have disease prevention properties. Afr. J. Biotechnol. 4(5):381-388.

Lebreton P, Jay M, Voirin B (1967). L'analyse qualitative et quantitative des flavonoïdes. Chim. Anal. Fr. 49(7):375-383.

Lybbert TJ (2007).Patente disclosure requirements and benefit sharing: A counterfactual case of Morocco's argan oil. Ecol. Econ. 64:12-18.

Lybbert TJ, Aboudrare A, Chaloud D, Magna N, Nash M(2011).Booming markets for Morroan argan oil appear to benefit some rural households while threatening the endemic argan forest. Proc. Natl. Acad. Sci. USA. 108:13963-13968.

Macías FA, Marín D, Oliveros-Bastidas A, Varela RM, Simonet AM, Carrera C, Molinillo JM (2003). Allelopathy as a new strategy for sustainable ecosystems development. Biol. Sci. Space 17(1):18-23.

Matern V, Grimming B, Kneusel RE (1995). Plant cell wall reinforcement in the disease-resistance response: molecular composition and regulation. Can. J. Bot. 73(S1):511-517.

Moiseev DV, Buzuk GN, Shelyuto VL (2011). Identification of flavonoids in plants by HPLC. Pharm. Chem. J. 45(1):35-38.

Neu R (1956). Ein neues reagenz zum nachweis und zur unterscheidung von flavonen impapier chromatogramm. Die naturwissenschaften, P. 43.

Reuber S, Bornman JF, Weissenböck G (1996). A flavonoid mutant of barley (*Hordeum vulgare* L.) exhibits increased sensitivity to UV-B radiation in the primary leaf. Plant Cell Environ. 19(5):593-601.

Ryan KG, Swinny EE, Markham KR, Winefield C (2002). Flavonoid gene expression an UV photoprotection in transgenic and mutant Petunia leaves, Phytochemistry 59:23-32.

Sebaa H, Kaid-Harche M (2014). Anatomical structure and ultrastructure of the endocarp cell walls of *Argania spinosa* (L.) Skeels (Sapotaceae). Micron 67:100-106.

Stapleton AE, Walbot V (2004). Flavonoids can protect maize DNA from the induction of ultraviolet radiation damage, Plant Physiol.105:881-889.

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Evaluation of the anti-inflammatory properties of the hexane extract of *Hydrocotyle bonariensis* Comm. Ex Lam. leaves

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This study investigates the anti-inflammatory properties of *Hydrocotyle bonariensis* Comm. Ex Lam, a medicinal plant used by indigenous traditional healers to manage chronic inflammatory diseases especially rheumatism and arthritis. The hexane extract of *H. bonariensis* leaves was evaluated for the presence and concentration of phytochemicals. It was subjected to heat-induced albumin denaturation and human red blood cell (HRBC) membrane stabilization assays. The anti-inflammatory properties of the extract were further assessed by employing the formaldehyde induced arthritis animal model assay. The extract was finally subjected to GC/MS analysis for the tentative identification of the phytochemical constituents. Phytochemical analysis of the extract revealed the presence of saponin, phenol, flavonoid, tannin, terpenoid and sterol. This extract showed the ability to inhibit thermally-induced protein denaturation and stabilize HRBC membrane in concentration dependent manner. In the formaldehyde induced arthritis model, the paw measurements of the rats were taken, their hematological parameters were determined, and their liver function tests (aspartate aminotransferase and alanine aminotransferase activities) were also carried out. Results from the animal model indicated that oral administration of the hexane extract of *H. bonariensis* leaf at a dose of 250 mg/kg body weight had potent anti-inflammatory action. The GC/MS analysis suggested a number of anti-inflammatory compounds in the extract among which were: hexadecanoic acid methyl ester, falcarinol and phytol. Consequently, the tentative identification of phytochemicals with anti-inflammatory activity in *H. bonariensis* affirms the anti-inflammatory property of the plant and the phytochemicals could serve as lead compounds for designing anti-inflammatory drugs.

Key words: *Hydrocotyle bonariensis*, anti-inflammatory, phytochemicals, protein denaturation, membrane stabilization, arthritis.

INTRODUCTION

Inflammation is a very complex response that occurs as a result of an injury, infection or another stimulus, in which several cell types and secreted factors elicits protective immunity, tissue repair and resolution of tissue damage (Howcroft et al., 2013). It is characterized by five pathological phenomena; *calor*-increase in tissue temperature, *rubor*-redness of vascularized tissue at inflammation site, *dolor*-intensive sensation of a noxious stimulus, *tumor*-swelling of the tissue, *functio laesa*-impaired function of the affected organ (Rather, 1971), all

of which are secondary to the direct consequence of tissue injury-enhancement of vascular permeability, protein denaturation and membrane alteration (Ryan and Majno, 1977; Majno and Plalade, 1961; Umapathy et al., 2010). It is worthy of note that though inflammatory responses are part of the body's defense mechanism, therefore important and useful, its prolonged action or escalation may cause debilitating harm to the body (Stankov, 2012). Chronic inflammation triggers casual pathways associated with aging, including physical frailty, energy imbalance, homeostatic dysregulation, changes in body composition and neuro-degeneration (Howcroft et al., 2013). Most currently used drugs for the treatment of inflammation-related diseases are the steroidal and non-steroidal drugs. These drugs have remarkable potency, however, long-term administration is required for treatments of chronic diseases. In addition, these drugs are known to have several adverse effects, and this has encouraged the use of medicinal plants with very little side effects to substitute for these chemical therapeutics.

The use of plants as medicine predates written human history itself but documentation can be found as far back as approximately 6000 years (Paulsen, 2010). Medicinal plants play an important role in the development of potent therapeutic agents and have contributed significantly towards the development of modern medicine. In recent times, use of medicinal plants worldwide was re-evaluated by extensive research on different plant species and their active therapeutic principles and it was concluded that the wealth of the plant kingdoms can represent novel sources of newer compounds with significant anti-inflammatory activities. Major advantages of the use of plants as medicine over conventional drugs seem to be their perceived efficacy, low incidence of serious adverse effects, and low cost (Sangita et al., 2012).

Hydrocotyle bonariensis, commonly known as large leaf pennywort is a member of the family *Araliaceae* and genus *Hydrocotyle*. It is a herbaceous plant with prostate, creeping or floating stems and roots forming at nodes. There are approximately 402 species in this genus and they are mostly found in Africa and America. Its common name in West Africa, Nigeria is Karo (Ajani, 2012). It is well known for its traditional uses and medicinal properties for the treatment of various kinds of diseases such as tuberculosis, relieving the pains of inflammation, rheumatism and arthritis, to increase brain capacity and for longevity (Masoumian et al., 2011). This herb has its medicinal uses as emetics, diuretics and laxatives (Evans, 1992). Leaves of this plant have been reported to contain alkaloids, flavonoids, tannins, phenolics and saponins (Ajani, 2012). The anti-mutagenic activity of the aqueous and methanolic extracts from the leaves and stems has been reported (Florinsiah et al., 2013). Results obtained in a study by Ajani (2012), suggested that chronic administration of *H. bonariensis* aqueous leaf extract may not contribute to liver and renal dysfunction. The study also indicated that when administered at an acute dose, the extract may not potentiate any significant toxic effect. It has also been reported that *H. bonariensis* possesses significant antioxidant property that may offer protection from galactose induced oxidative damage in both the lens and the liver (Ajani, 2012).

To the best of our knowledge, no attempts have been made to evaluate the anti-inflammatory properties of the hexane extract of *H. bonariensis* leaves. However, Ouviña et al. (2009) explored anti-inflammatory activity of an infusion and methanolic extract of aerial parts of H. bonariensis. Therefore, this study aimed at identifying the phytochemicals present in the hexane extract of *H. bonariensis* leaves, identify the bioactive agents in the extract using Gas Chromatography/Mass Spectrometry analytical method and evaluate the anti-inflammatory properties of the extract using some in *vitro* and in *vivo* methods, with the objective of assessing the therapeutic use of *H. bonariensis* in the management of inflammatory diseases.

MATERIALS AND METHODS

Collection of plant material, Identification and preparation of extract

Fresh leaves of *H. bonariensis* were collected from a garden in Ilishan-Remo, Ogun State, and authenticated by a plant taxonomist, Prof E. B. Esan of the Department of Basic Sciences, Babcock University Ilishan-Remo, Ogun State of Nigeria. The leaves were washed with tap water to remove all debris, air-dried at room temperature (25°C) for four weeks and pulverized mechanically, using a warring blender. The extract was prepared according to the method of Florinsiah et al. (2013). 80 g of the dry powdered plant material was soaked in 800 ml of n-hexane with intermittent shaking for 48 hours and filtered through Whatman No. 1 filter paper. The filtrate was concentrated at 45°C using rotary evaporator (RE52-3 model, LIDA Instrument).

Preliminary screening of the extract phytochemicals

Initial screening tests of the extract were performed to ascertain the presence or absence of phenolics, flavonoids, saponins, tannins, terpenoids and sterols using standard procedures at 45°C

Determination of total phenolic content

Total phenolic content in the extract was determined according to the method of Singleton et al. (1999). An aliquot of 0.5 ml of extract (1mg/ml) was mixed with 2.5 ml Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 2 ml (7.5% w/v) of sodium

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carbonate (Na_2C0_3). The tube was vortexed for 15 s and incubated for 40 min at 45°C for colour development. Absorbance was measured at 765 nm using double beam UV/visible spectrophotometer (T80 model, PG Instrument). A calibration curve was obtained using gallic acid as standard, the total phenolics content was expressed as mg/g gallic acid equivalent using the equation obtained from the calibration curve.

Estimation of total flavonoids

The formations of a complex aluminum chloride were estimated using the method described by Ordonez et al. (2006). 0.5 ml of the extract (1 mg/ml) was mixed with 0.5 ml of 2% aluminum chloride ($AlCl_3$) prepared in ethanol. The resultant mixture was incubated for 60 min at room temperature for yellow colour development to indicate the presence of flavonoid. Absorbance was measured at 420 nm using double beam UV/visible spectrophotometer (T80 model, PG Instrument). A calibration curve was obtained using quercetin as standard, and the total flavonoids content was expressed as mg/g quercetin equivalent using the equation obtained from the calibration curve.

Determination of tannin concentration

Tannin content of the sample was determined according to the modified vanillin-HCl methanol method as described by Noha et al. (2011). The vanillin-HCl reagent was prepared by mixing an equal volume of 8% HCl and 1% vanillin in methanol. The reagent was mixed just prior to use. About 0.2 g of the ground sample was placed in a small conical flask. 10 ml of 1% concentrated HCl in methanol was added. The flask was capped and continuously shaken for 20 min and the content was further centrifuged at 2500 rpm for 5 min with 1.0 ml of the supernatant was pipetted into a test tube containing 5 ml of vanillin-HCl reagent. Absorbance at 450 nm was read on the spectrophotometer (T80 model, PG Instrument) after 20 min of incubation at 30°C. A standard curve was prepared to express the result as tannic acid equivalent; tannin (%) = C × 10 × 100/200. Where C = concentration corresponding to the optical density; 10 = volume of the extract (ml); 200 = sample weight (mg).

Determination of saponin concentration

5 g of plant sample was dispersed in 50 ml 20% v/v ethanol prepared in distilled water. The suspension was heated over a hot water bath for 1 h with continuous stirring at 55°C. The mixture was filtered and the residue re-extracted with another 50ml of 20% ethanol. The combined extracts were reduced to 20 ml over hot water bath at about 90°C. The concentrated solution obtained was shaken vigorously with 10 ml of diethyl ether in a 250 ml separating funnel; the aqueous layer was collected while the ether layer was discarded. 20 ml of but-1-ol was added to the filtrate and washed three times with 10 ml of 5% w/v aqueous sodium chloride. The whole mixture was heated to evaporation on a hot water bath and later oven-dried to a constant weight. The percentage saponins content of the sample was calculated using the formula described by Okwu and Josiah (2006).

% Saponins = Weight of final filtrate/weight of sample × 100

Gas chromatography/mass spectrometry (GC/MS) analysis

GC/MS analysis was carried out at the Department of Chemistry, University of Lagos Akoka, by using Agilent Technology model 7890A GC/MS, MSD = 5975C (detector) Agilent Technologies, Injector: 7683B Series, Initial temperature = 100°C held for 2 min, final temperature = 270°C at the rate 10°C/min, 1 µl of the hexane extract of *H. bonariensis* leaves was injected. Temperature of heater was 250°C, pressure was 3.2652 psi, mode type slit less, column type (HP 5MS: 30 m x 320 µm x 0.25 µm) and carrier gas (Helium, 99.9999% purity, flow rate = 1.4963 ml/min; average velocity = 45.618 cm/s). The constituent compounds were determined by comparing their retention times and mass weights with those of authentic samples obtained by GC as well as the mass spectra National Institute of Science and Technology (NIST) version 2.0 MS database and literatures.

Human red blood cell (HRBC) membrane stabilization method

Estimation of *in vitro* anti-inflammatory activity by HRBC membrane stabilization method described by Sadique et al. (1989) was used to assess the hexane extract *in-vivo* anti-inflammatory effect. The principle involved is stabilization of the human red blood cell membrane by hypotonicity-induced membrane lysis. The assay mixture containing 1 ml phosphate buffer (pH 7.4, 0.15 M), 2 ml hypo saline (0.36%), 0.5 ml HRBC suspension (10% v/v) with 0.5 ml of the plant extract of various concentrations 50 to 250 µg/ml; standard drug diclofenac sodium 50 to 250 µg/ml and control (distilled water instead of hypo saline to produce 100% haemolysis) were incubated at 37°C for 30 min followed by centrifugation at 3000 rpm for 10 min. The hemoglobin content in the suspension was estimated using double beam UV- visible spectrophotometer (T80 model, PG Instrument) at 560 nm. The percentage haemolysis produced in the presence of distilled water was taken as 100%. Percentage of HRBC membrane stabilization or protection was calculated using the formula,

Percent stabilisation = 100 – (optical density of drug) ÷ (optical density of control) × 100

Preparation of red blood cells (RBCs) suspension

Fresh whole human blood was collected from a healthy human volunteer who had not taken any None Steroidal Anti-Inflammatory Drugs (NSAIDs) for two weeks prior to the experiment and centrifuged at 3000 rpm for 10 min. It was washed four four times with equal volume of normal saline. The volume of blood was measured and re constituted as 10% v/v suspension with normal saline Sadique et al. (1989).

Inhibition of protein denaturation assay

The effect of the plant extract on protein denaturation was studied using the method of Sakat et al. (2010), with minor modifications. The reaction mixtures in test tubes contained 50 µl of various studied concentrations 10 - 80 µg/ml of diclofenac sodium and test fractions and 50 µl methanol as a control. 450 µl of 5% w/v bovine serum albumin (BSA) was added to the above test tubes. The test tubes were incubated at 37°C for 20 min and then heated at 57°C for 10 min. After cooling, 2.5 ml phosphate buffered saline (pH 6.3) was added to each tube. The absorbance of these solutions was measured using double beam UV/visible spectrophotometer (T80 model, PG Instrument) at wavelength 660nm. The percent inhibition of denaturation was calculated as follows:

% inhibition = (Absorbance of control – Absorbance of treated)/Absorbance of control x 100

Experimental animals and care

120 to 200 g male albino rats (Wistar strain) purchased from

Babcock University animal house, Ilishan-Remo, Ogun State were used for the study. They were acclimatized for two weeks in the animal house; Babcock University Illishan-Remo Ogun State. The animals were kept in aerated wooden cages under a natural light condition at room temperature (28 to 30°C) and fed with rat cubes (pellets) and water ad *libitum*. All the animal experiments and protocol conformed to the guidelines of National Institute of Health (NIH, 2000) for laboratory animal care and use.

Formaldehyde-induced arthritis assay

The protocol described by Eun-Michoi and Jae-Kwan (2004), was used for this experiment. Thirty (30) rats were divided based on their weights into six groups of five rats each. Arthritis was induced by injecting 0.02 ml formaldehyde (2% v/v) solution into the left hind paw of the rats; beneath the plantar aponeurosis on the first and third days 30 min after the oral administration of the standard drug and varied doses of the test extract and the administration of the drug and the extract continued for seven days.

Experimental design

Group I: Normal rats that were not induced with arthritis and given 0.5 ml of normal saline.
Group II: Control (untreated) rats that were induced with arthritis and given 0.5 ml of normal saline.
Group III: Arthritis induced rats that were treated with diclofenac sodium, a NSAID; (10 mg/kg) body weight.
Group IV: Arthritis induced rats that were treated with the hexane extract of *H. bonariensis* leaves (50 mg/kg) body weight.
Group V: Arthritis induced rats that were treated with the hexane extract of *H. bonariensis* leaves (100 mg/kg) body weight.
Group VI: Arthritis induced rats that were treated with the hexane extract of *H. bonariensis* leaves (250 mg/kg) body weight.

Measurement of paw thickness

The paw thickness of the rats was measured using a micrometer screw gauge for seven days. The change in paw size was obtained by subtracting the baseline values from values obtained during the period of the experiment. The baseline values are the values from measurements taken before the induction of arthritis.

Sacrificing of animal

Rats were made to fast overnight the seventh day, they were euthanized by cervical dislocation before being sacrificed, and blood was collected by cardiac puncture into lithium heparinised tubes for liver function tests (AST and ALT determination) and into EDTA tubes for the determination of hematological parameters.

Collection of plasma

Plasma for liver function tests was obtained by centrifuging the collected blood samples at 3000 g for 10 min.

Determination of plasma alanine amino transferase (ALT)

Assay of alanine aminotransferase (ALT) was carried out using the procedure provided by the Randox kit manufacturer and followed the principle described by Reitman and Frankel (1957).

Table 1. Phytochemicals identified in the hexane extract of *H. bonariensis* leaf.

Phytochemical	Presence	Concentration
Phenol	+	1.07 ± 0.003 mg(GE)/g
Flavonoid	+	1.36 ± 0.042 mg(QE)/g
Tannin	+	18.74 ± 0.050% *
Saponin	+	47.20 ± 1.600% *
Terpenoid	+	ND
Sterol	+	ND

Values are expressed as means ± standard deviation of the mean (n = 3). + Presence of phytochemicals; GE: Gallic acid Equivalent; QE: Quercetin Equivalent; *Phytochemicals having concentrations evaluated from the ground sample. ND: Not determined.

Determination of plasma aspartate amino transferase (AST) activity

The activity of AST was assayed using the procedure provided by the Randox kit manufacturer and followed the principle described by Reitman and Frankel (1957).

Determination of hematological parameters

The test to determine the hematological parameters of the rats was carried out at the hematological unit of Babcock University Teaching Hospital (BUTH). It was done using an auto-analyzer (Swelab Alfa 3- Part Hematology Analyzer by Boule Medicals).

Statistical evaluation

Unless otherwise stated, all values are expressed as mean ± standard deviation of triplicate readings. Where applicable, statistical evaluation was done using one way analysis of variance (ANOVA) followed by Duncan's Post Hoc Test (DPHT). The significance level was set at $P<0.05$.

RESULTS

Phytochemical analysis

The result of the phytochemical screening of the hexane extract of *H. bonariensis* leaves presented in Table 1 reveals the presence of terpenoid, steroids, phenol, flavonoid, tannin and saponin.

GC/MS analysis

Hexadecanoic acid methyl ester, falcarinol and phytol which are compounds that have been reported to exert anti-inflammatory effects were three of the major compounds suggested by the GC/MS analysis to be present in the hexane extract of *H. bonariensis* leaves (Table 2, Figure 1).

Table 2. Bioactivity of the compounds detected in the hexane extract of *H. bonariensis* leaf by the GC/MS.

Bioactive agent	Peak #	Retention time (min)	Area %	Reported bioactivity	References
Cyclohexasilo-xane, dodecamethyl (Siloxane)	1	15.149	1.00	Antifungal properties	Mahmoud et al. (2013)
Caryophyllene (Terpenoid)	2	18.124	1.03	Anti-inflammatory, anti-carcinogenic, local anaesthetic activities, antioxidant, antibiotic	Legault and Pichette (2007)
1,3,6,10- Dodecatetraene,3,7,11-trimethyl-, (Z,E)- (Sequisterpene)	3	18.611	1.02	Insect semiochemical; pheromones	Sobotnik et al. (2008); Hern and Dorn (1999)
3,5-Octadiene, 2,2,4,5,7,7- hexamethyl-, (E,Z)-	4	19.349	8.90	No reported bioactivity	
Spiro[5.5]undeca-1,8-diene,1,5,5,9-tetramethyl-,	5	20.138	1.27	No reported bioactivity	
Cycloheptasiloxane, tetradecamethyl-(Siloxane)	6	20.482	1.97	Antifungal properties	Mahmoud et al. (2013)
Caryophyllene oxide (Terpenoid)	7	23.068	1.47	Antifungal; Flavouring/Fragrance ingredient; Antibacterial, antitumor, anti-inflammatory	Yang et al. (1999); Antonella et al. (2013); Egharevba et al. (2012)
Silane,[[4[1,2bis [(trimethylsilyl) oxy]ethyl]-1,2-phenylene]bis(oxy)] bis[trimethyl-	8	25.334	2.37	No reported bioactivity	
5-Isopropyl-2-methylphenyl 3- methyl butanoate	9	26.570	0.70	No reported bioactivity	
Cyclononasiloxane, octadecamethyl	10, 15, 23, and 29	29.557, 33.333, 41.293, and 49.681	1.55, 1.42, 1.65, and 1.95	No reported bioactivity	
Bicyclo[3.1.1]heptane,2,6,6-trimethyl-, (1.alpha.,2.beta.,5 alpha.)	11	30.112	26.39	No reported bioactivity	
2-pentadecanone, 6, 10, 14-trimethyl	12	30.181	1.24	Antibacterial	Nurettin et al. (2006)
Bicyclo [4.1.0] heptane,3-methyl	13	30.564	0.74	No reported bioactivity	
Hexadecanoic acid,methyl ester	14	32.143	3.49	Anti-inflammatory	Cai et al. (2005)
Falcarinol	16 and 17	34.998 and 35.141	6.08 and 7.58	Anti-inflammatory; antioxidant; anticancer; antifungal; natural pesticide	Kobaek-Larsen et al. (2005); Christensen (2009)
9,12- Octadecadienoic acid,methyl ester	18	36.355	3.21	Influence synthesis of prostaglandins and other cell regulators	Magdi et al. (2009)
9,12,15- Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	19	36.555	3.18	Anti-inflammatory	Lalitharani et al. (2009)
Phytol (diterpene)	20	37.133	11.09	Transcription modulator, anti-inflammatory, fragrance	Gloerich (2005); Silva et al. (2013);IFRA (2004)
Hexasiloxane, tetradecamethyl-	21, 26, and 28	37.367, 44.382, and 46.992	1.84, 1.71, and 1.77	No reported bioactivity	
Tetracosane	25	43.988	0.72	Cytotoxicity activities	Uddin et al. (2012)
Hexadecane	27	45.893	0.57	Antibacterial, antioxidant	Yogeswari et al. (2012)
2,6,10,14,18,22- Tetracosahexane (Isomer of squalene ; a triterpene)	30	49.950	1.98	Intermediate in the biosynthesis of cholesterol, emmollient for skin, antioxidant, antitumor activities	Zih-Rou et al. (2009)

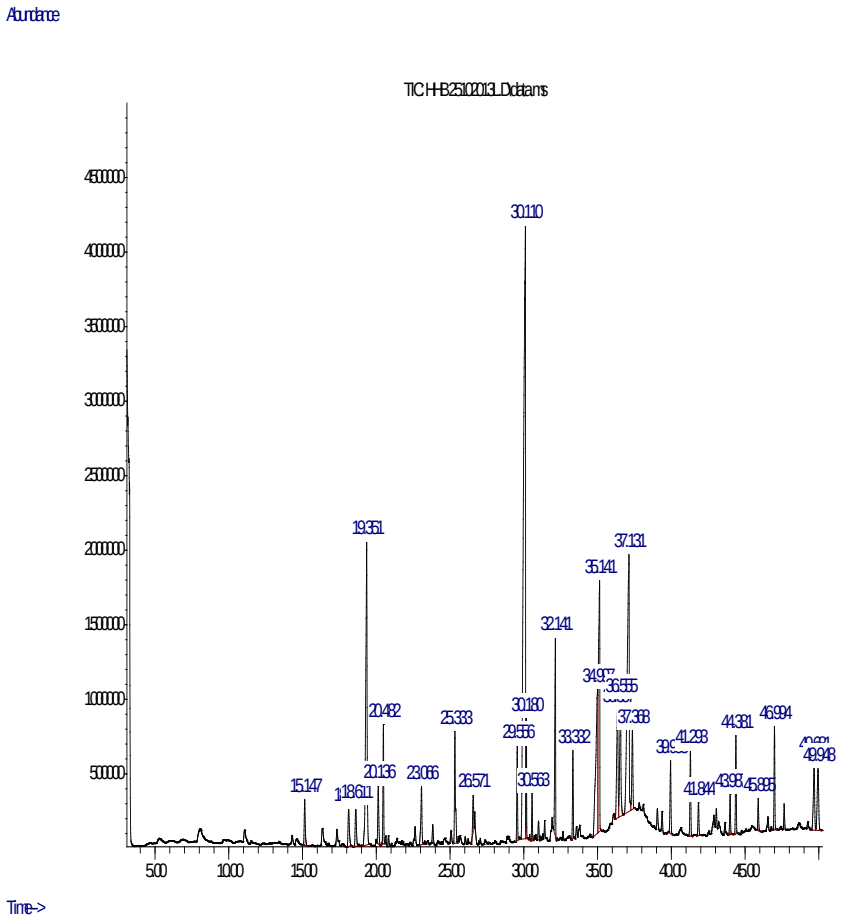

Figure 1. GCMS chromatogram of the hexane extract of *H. bonariensis* leaf.

Membrane stabilization

The hexane extract of *H. bonariensis* leaf showed stabilization with increase in concentration (30.16 ± 0.95 to 71.32 ± 0.44%) and IC_{50} value of 117.37 µg/ml. The standard drug, diclofenac sodium used as reference also did the same (52.65 ± 0.85 to 95.63 ± 0.44%) with an IC_{50} value of 47.34 µg/ml (Figure 2).

Inhibition of protein denaturation

In this study, the hexane extract of *H. bonariensis* leaves was found to inhibit protein denaturation in a concentration-dependent manner (26.50 ± 6.45 to 69.23 ± 5.12)% with an IC_{50} value of 44.84 µg/ml compared to standard drug diclofenac sodium which also did the same (46.15 ± 3.63 to 85.47 ± 2.96%) with an IC_{50} value of 10.68 µg/ml (Figure 3).

Animal studies

The comparative inhibitory effect of the hexane extract of *H. bonariensis* leaf and standard drug, diclofenac sodium on edema induced by the formaldehyde injection which was measured by change in paw thickness of the rats is presented in Figure 4. From the result, 250 mg/kg body weight oral administration of the hexane extract of *H. bonariensis* leaf showed significant ($P<0.05$) reduction in the induced edema starting from the fourth day when compared to the control (untreated), thereby suggesting the anti-inflammatory action at this concentration. No significant reduction of the edema was observed from the administration of (50 and 100) mg/kg body weight of the extract.

The results from the hematological parameters shown in Figures 5 to 9 present the total White Blood Cell (WBC), the differential white cell count (lymphocytes, granulocytes, eosinophils, monocytes and basophils) and the total platelet count. From the results, consistency in reduction of these cells was indicated in the group of rats that were orally administered 250 mg/kg body weight hexane extract of *H. bonariensis* leaf, when compared to the control. Figures 10 and 11 present the results of the liver function tests (AST and ALT activity) of the rats. From the results oral administration of 250 mg/kg body weight of the hexane extract of *H. bonariensis* leaf

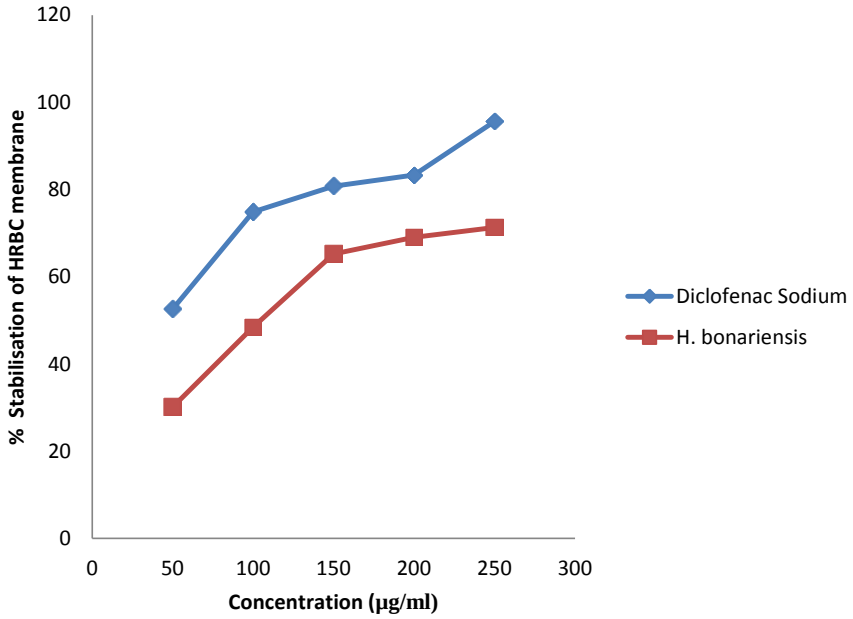

Figure 2. Percentage stabilization of human red blood cell (HRBC) membrane with diclofenac sodium and *H. bonariensis extract.* IC_{50} of diclofenac sodium = 47.34 ug/ml; IC_{50} of *H. bonariensis* leaf (hexane extract) = 117.37 µg/ml.

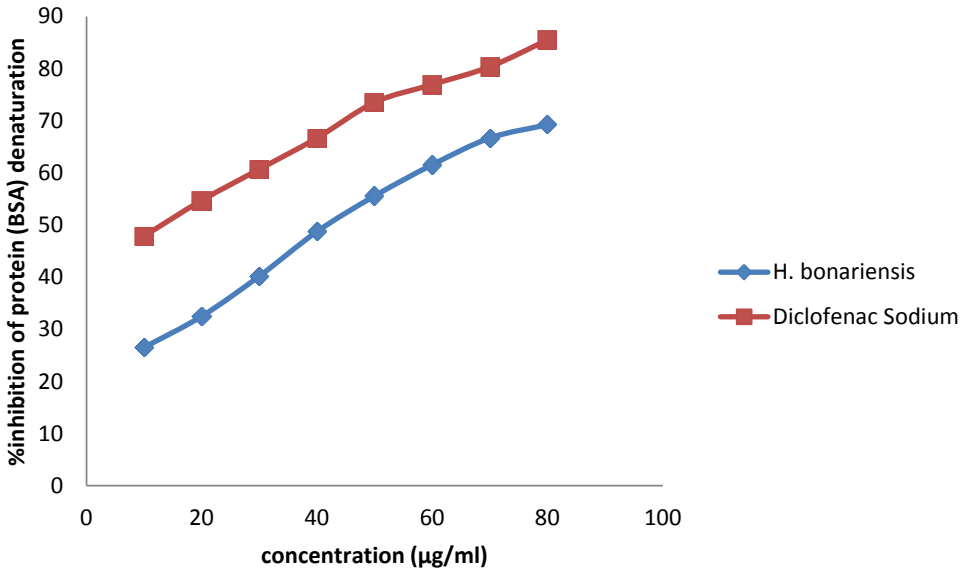

Figure 3. Percent inhibition of the denaturation of protein (BSA) by the hexane extract of *H. bonariensis* leaf and standard drug, diclofenac sodium. IC_{50} of diclofenac sodium = 10.68 µg/ml; IC_{50} of *H. bonariensis* leaf (hexane extract) = 44.84 µg/ml.

showed a significant reduction ($P<0.05$) in AST and ALT activities in the rats' blood plasma when compared to the control. Oral administration of the extract at doses of 50 and 100 mg/kg body weight did not show a significant reduction ($P>0.05$) in the activities of the enzymes.

DISCUSSION

The search for phytochemicals possessing anti-inflammatory properties has been on the rise due to their potential use in the therapy of various chronic and

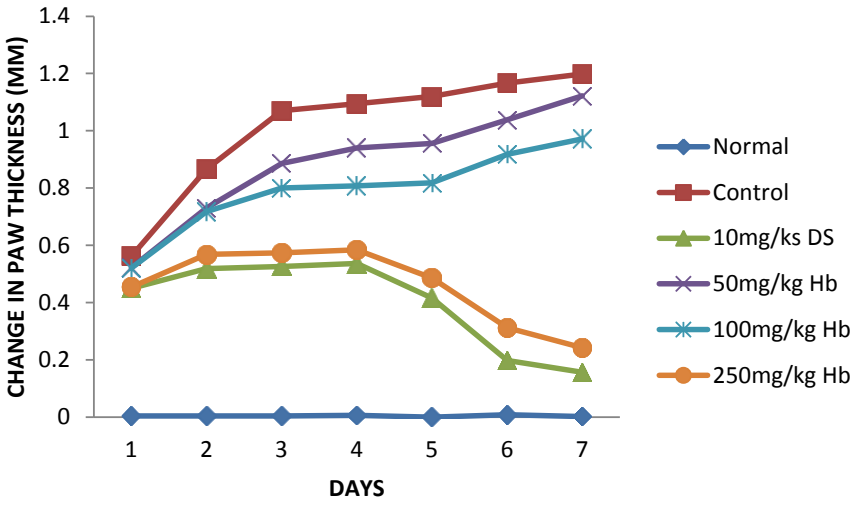

Figure 4. Comparative inhibitory effect of the hexane extract of *H. bonariensis* leaf and standard drug, diclofenac sodium on edema induced by the formaldehyde injection.

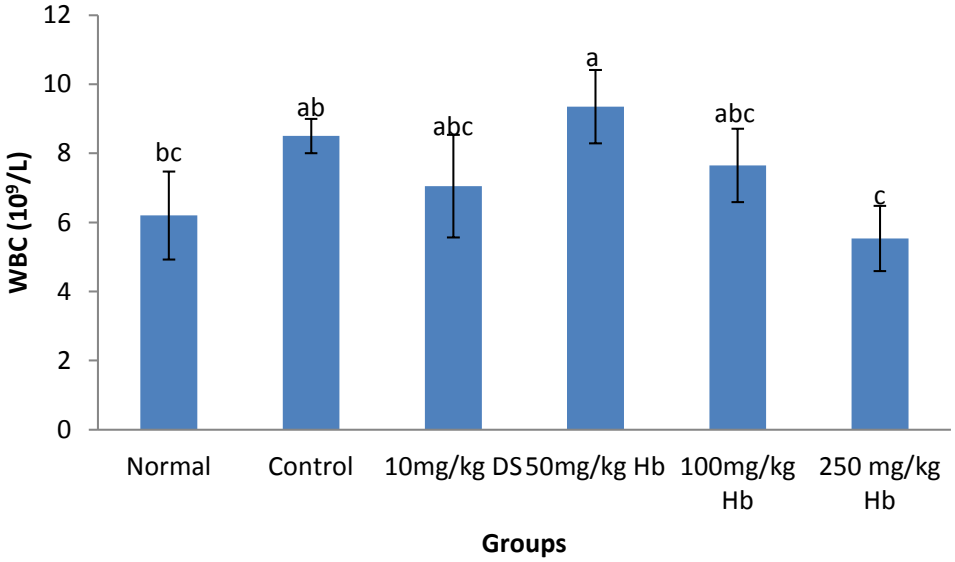

Figure 5. Total white blood cell (WBC). Values are means ± standard deviation (n = 5); bars with different alphabets are significantly different ($P < 0.05$).

compounds have been found to possess potent anti-inflammatory activity (Sakat et al., 2010; Roy et al., 2010; Garg et al., 2010). Also, several authors have reported the anti-inflammatory properties of flavonoids in various studies (Antonella et al., 2013; Lopez-Lazaro, 2009; Yoshida et al., 2008; Amarlal et al., 2009). Tannins have been reported to have potent anti-inflammatory properties (Souza et al., 2007; Fawole et al., 2010). The percentage of saponin in *H. bonariensis* leaf was found to be 47.20 ± 1.60%. Saponins are well known to have anti-inflammatory activity (Uddin et al., 2012). Thus, the major part of the anti-inflammatory properties of *H. bonariensis* could be due to the saponin content. The phytochemicals detected in this study are in agreement with those reported by Ajani (2012).

Hexadecanoic acid methyl ester, falcarinol and phytol which are compounds that have been reported to exert anti-inflammatory effects were three of the major compounds suggested in the hexane extract of *H. bonariensis* leaves. In studies with isolated kupffer cells treated with lipopolysaccharide (Cai et al., 2005), Hexadecanoic acid methyl ester showed anti-

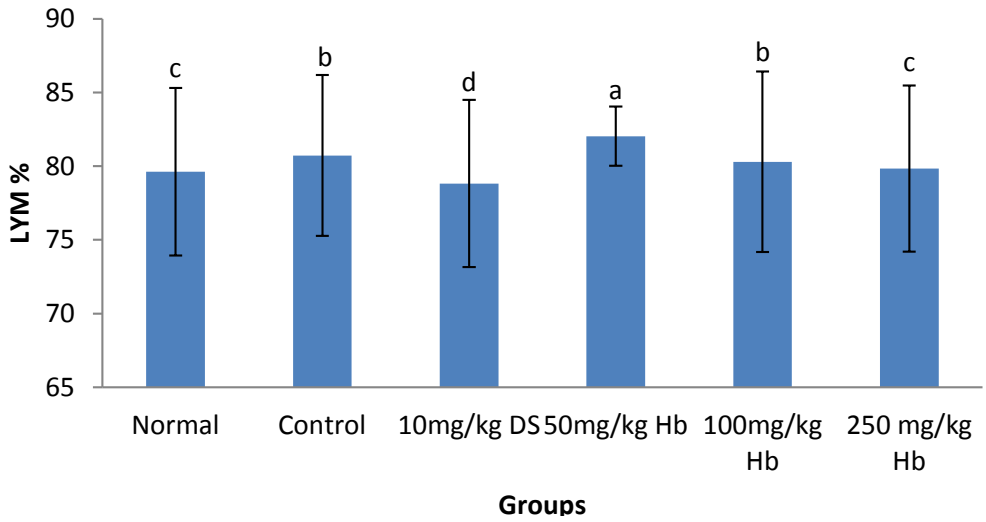

Figure 6. Percent lymphocytes (LYM). Values are expressed as means ± standard deviation (n = 5); bars with different alphabets are significantly different ($P < 0.05$).

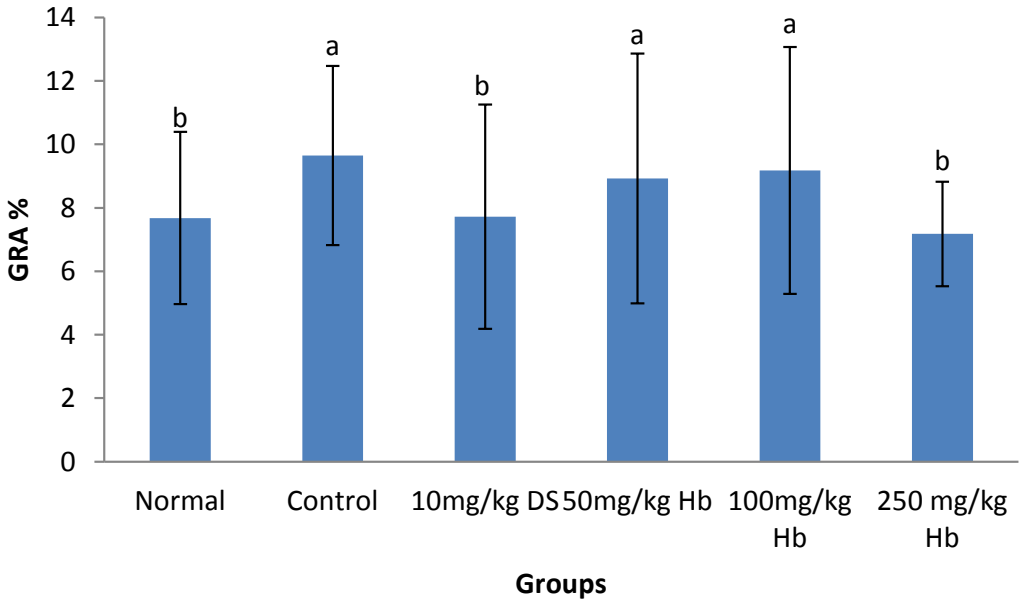

Figure 7. Percent granulocytes (GRA). Values are expressed as means ± standard deviation (n = 5); bars with different alphabets are significantly different ($P < 0.05$).

1467 inflammatory activity by decreasing secretion of interleukin-10, tumor necrosis factor-α (TNF-α), nitric oxide, and prostaglandin E_2. In a recent study by Silva et al. (2013), phytol was reported to inhibit the recruitment of total leukocytes and neutrophils; decreased MPO (myeloperoxidase) activity, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) levels, and MDA (malondialdehyde) concentration during carrageenan-induced acute inflammation. The study thus suggested that phytol attenuates the inflammatory response by inhibiting neutrophil migration which is partly done by the reduction in IL-1β and TNF-α levels. The anti-inflammatory mechanism of falcarinol is still unclear, however the reactivity of falcarinol towards mercapto and amino groups in proteins forming a hapten-protein complex (antigen), probably due to its hydrophobicity and its ability to form an extremely stable carbocation with the loss of water, thereby acting as a very reactive alkylating agent towards various biomolecules could explain its anti-inflammatory mechanism (Alan et al., 2006).

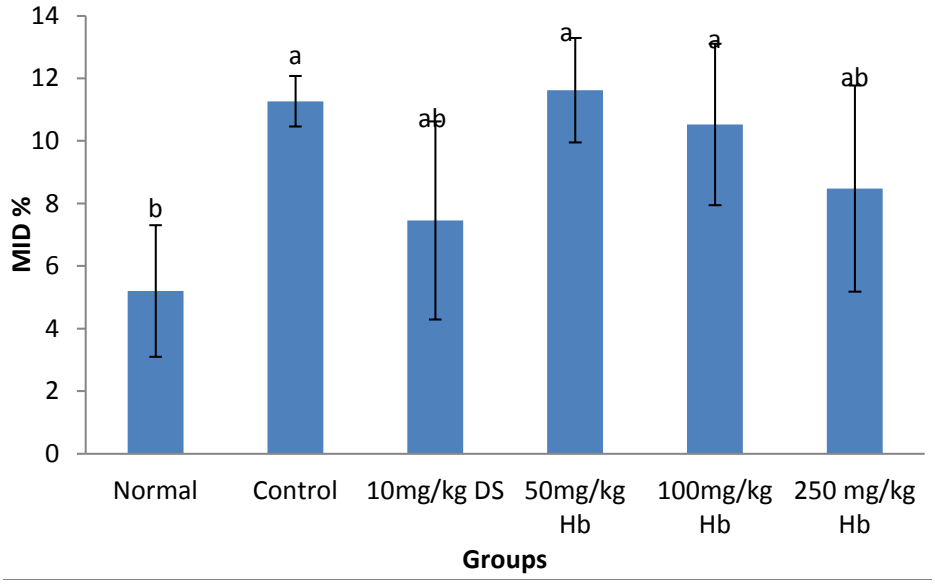

Figure 8. Percent eosinophils, monocytes and basophils (MID). Values are expressed as means ± standard deviation (n = 5); bars with different alphabets are significantly different ($P < 0.05$).

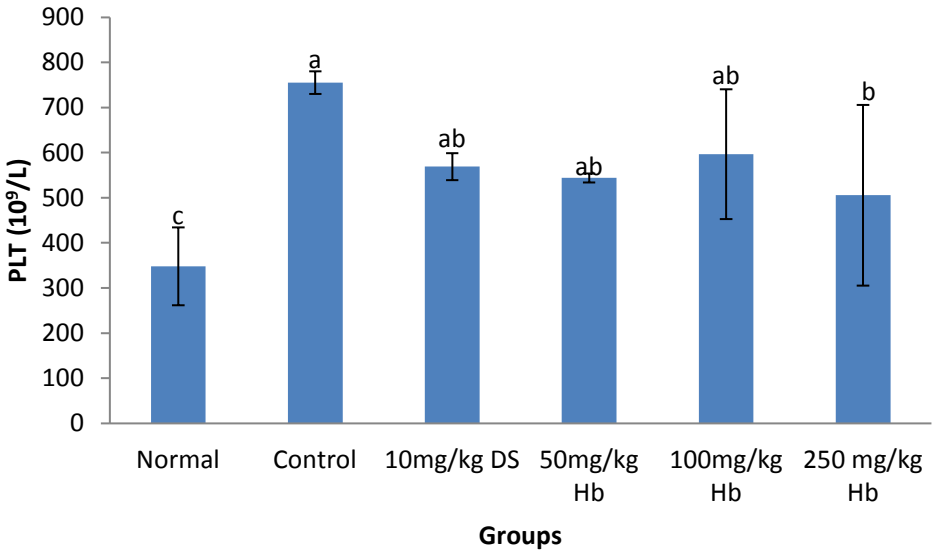

Figure 9. Total platelet count (PLT). Values are expressed as means ± standard deviation (n = 5); bars with different alphabets are significantly different ($P < 0.05$).

The precise mechanism of membrane stabilization is yet to be elucidated. However, this effect could be possible when surface area/volume ratio of cells are brought about by an expansion of the membrane or the shrinkage of the cells and an interaction with membrane proteins (Shinde et al., 1999). The HRBC membrane stabilization has been used as a method to study the in *vitro* anti-inflammatory activity (Chou, 1997). Stabilization of the erythrocyte membrane is important in limiting the anti-inflammatory response because it prevents the release of lysosomal constituents of activated neutrophils such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon extracellular release (Chou, 1997). Some NSAIDs are known to possess membrane stabilization properties which may contribute to the potency of their anti-inflammatory effect. These nonsteroidal drugs act either by inhibiting these lysosomal enzymes or by stabilizing

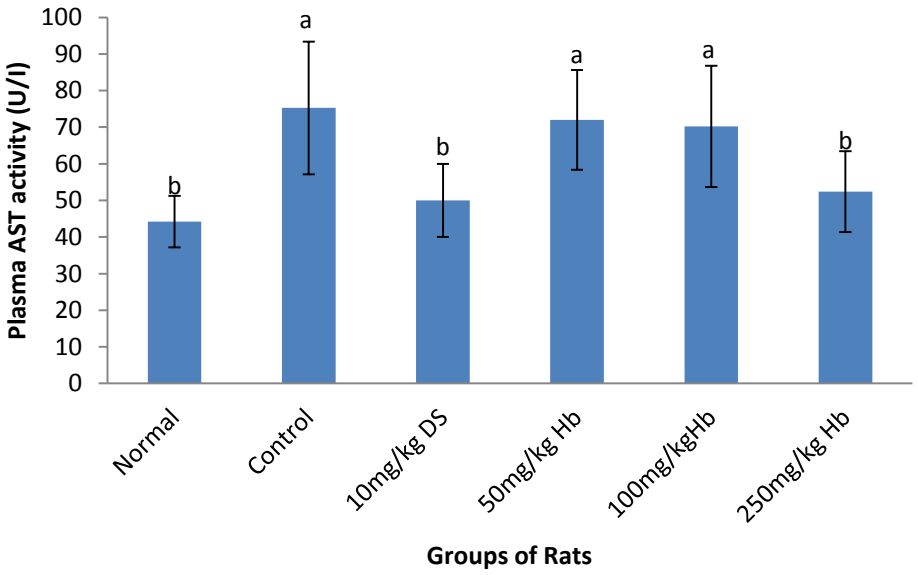

Figure 10. Plasma aspartate aminotransferase (AST) activity (UI). Values are expressed as means ± standard deviation (n = 5); bars with different alphabets are significantly different ($P < 0.05$).

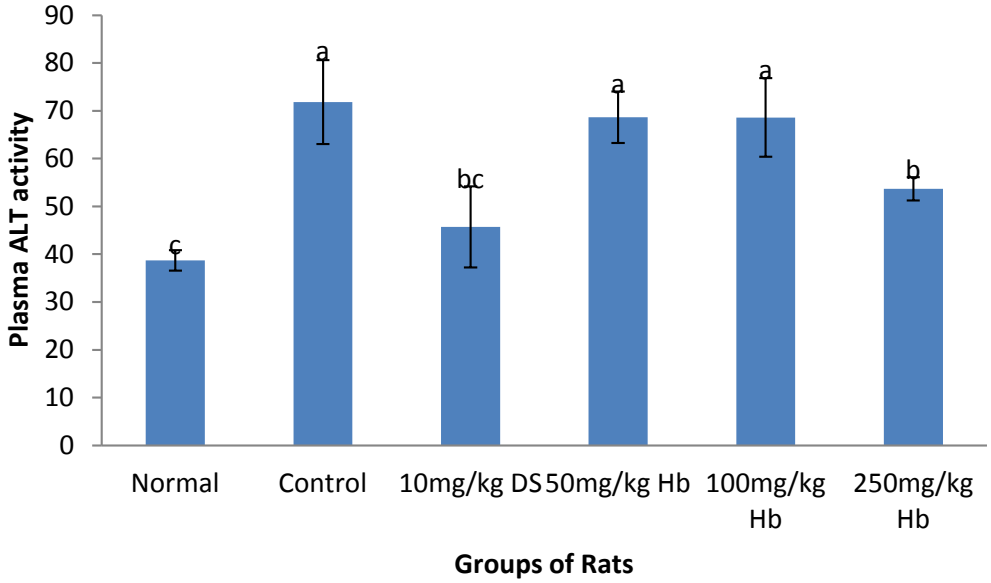

Figure 11. Plasma alanine aminotransferase (ALT) activity (UI). Values are expressed as means ± standard deviation (n = 5); bars with different alphabets are significantly different ($P < 0.05$).

the lysosomal membrane (Manjunatha et al., 2013). From the results shown in this study, *H. bonariensis* appears to possess membrane stabilization properties which may contribute to the potency of the anti-inflammatory effect of the plant.

Denaturation of tissue proteins is one of the well-documented causes of inflammatory and arthritic diseases. Production of auto antigens in certain arthritic diseases may be due to denaturation of proteins in *vivo* (Opie, 1962; Umapathy et al., 2010). Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug development. The anti-inflammatory drugs (salicylic acid, phenylbutazone etc), have shown dose-dependent ability to inhibit protein denaturation and similar results have been observed from many reports from plant extract (Sakat et al., 2010). The result thus agrees with the earlier reports. The extract could also possibly inhibit the release of the lysosomal

content of neutrophils at the site of inflammation (Chou, 1997).

The various NSAIDs exert their effects by the pharmacological inhibition of COX (cyclooxygenase enzyme), responsible for the formation of biological mediators called prostanoids which include prostaglandins, prostacyclin and thromboxane that give rise to pain and inflammation. The consistent reduction in the studied hematological parameters (total white blood cell count, granulocytes, lymphocytes, eosinophils, monophils, basophils and total platelet count), observed with the oral administration of 250 mg/kg body weight of the hexane extract of *H. bonariensis* leaf when compared to the control could suggest that it could inhibit the cyclooxygenase (COX) enzyme, thus relieving pain and inflammation.

Enzymes are the best markers of tissue damage because of their specificity and catalytic activity to the tissue (Sivakumar et al., 2007). Increased AST levels in the plasma of the control rats and (50 and100) mg/kg b.w hexane extract of *H. bonariensis* leaf treated rats may have been due to the leakage of this enzyme from hepatocyte cells as a result of hepatocellular damage (Xing-Jiu et al., 2006) caused as a result of the induction of arthritis. AST and ALT are membrane bound enzymes, thus the reduction in the plasma activity of these enzymes observed at the oral administration of 250 mg/kg body weight of the extract suggests its ability to stabilize the liver membrane, which could have prevented the leakage of these enzymes into the plasma. The results obtained in both of the liver function tests (AST and ALT) are in correlation with each other. This agrees with what has been reported by Xing-Jiu et al. (2006) that the amount of AST and ALT in the blood is directly related to the extent of the tissue damage.

Conclusion

Results from this study indicate that the hexane extract of *H. bonariensis* leaves could possess anti-inflammatory properties. These activities may be due to the occurrence of phytochemicals such as alkaloids, flavonoids, tannins, steroids, saponins, terpenoids and phenols present in the extract. The potent anti-inflammatory activity observed by the oral administration of the extract at a dose of 250 mg/kg body weight gives an idea that the compounds of the plant could be used as a lead compound for designing a potent anti-inflammatory drug.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES

Ajani EO (2012). Biochemical effect of *Hydrocotyl bonariensis* (large leave pennyworth) Comm Ex Lam (Apiaceae) leave extract on glactose model of caractogenesis. PhD Thesis. Olabisi Onabanjo University, Ago Iwoye, Nigeria.

Alan C, Mike NC, Hiroshi A (2006). Plant Secondary Metabolites, Occurrence, Structure and Role in the Human Diet. UK: Blackwell Ltd. P. 150.

Amarlal S, Mira L, Nogueira JM, Da Silva AP, Helena FM (2009). Plant extracts with anti-inflammatory properties-a new approach for characterization of their bioactive compounds and establishment of structure-antioxidant activity relationships. Bioorg. Med. Chem. 17(5):1876-1883.

Antonella DF, Maffei PH, Francesco CM, Grazia S, and Gabriela M (2013). Genotoxicity assessment of β-caryophyllene oxide. Regul. Toxicol. Pharmacol. 66(3):264-268.

Cai P, Kaphalia BS, Ansari GAS (2005). Methyl palmitate Inhibitor of phagocytosis in primary rat Kupffer cells. Toxicology 210:197-204.

Chou CT (1997). The anti-inflammatory effect of Tripterygium wilfordii Hook F on adjuvant induced paw edema in rats and inflammatory mediators release. Phytother. Res. 11:152-154.

Christensen LP (2009). Bioactivity of Polyacetylenes in Food Plants. In. Watson RR, Preedy VR Edition. Bioactive Foods in Promoting Health. Oxford Academic Press. pp. 85-306.

Egharevba HO, Kunle FO, Okwute SK, Okogun JI (2012). Chemical constituents of the Essential Oil from Laggera pterodonta (DC) Sch. Bip. From North-Central Nigeria. J. Appl. Pharm. Sci. 2(8):198-202.

Eun-Michoi C, Jae-Kwan H (2004). Anti-inflammatory, analgesic and antioxidant activities of the fruit of FoeniculumVulgare. Fitoterapia 75:557-565.

Evans JP (1992). The effect of local resource availability and clonal integration on ramet functional morphology in Hydrocotyle bonariensis. Oecologia 89:265-276.

Fawole OA, Amoo SO, Ndhlala AR, Light ME, Finnie JF, Van SJ (2010). Anti-inflammatory,anticholinesterase, antioxidant and phytochemical properties of medicinal plants used for pain-related ailments in South Africa. J. Ethnopahmacol. 127(2):235-241.

Florinsiah L, Farida ZMY, Nur SN, Norfazlina MN, Suziana ZC, Rajab NF (2013). Mutagenicity Effect of Hydrocotyle bonariensis Extracts in Salmonella/Microsome Assay. Int. J. Pharm. Sci. Rev. Res. 20(2):47-50.

Garg VKR, Jain M, Sharma PKR, Garg G (2010). Anti-inflammatory activity of Spinacia oleracea. Int. J. Pharma Prof. Res. 1(1):1-4.

Gloerich J (2005). A phytol-enriched diet induces changes in fatty acid metabolism in mice both via PPARalpha-dependent and -independent pathways. J. Lipid. Res. 46:716-726.

Govindappa M, Sadanada TS, Channabasava R, Vinay B, Raghavendra (2011). In vitro anti-inflammatory, lipoxygenase, xanthin oxidase, xanthine activity of Tecoma stans (L) juss. Ex Kunth. Int. J. Pharma BioSci. 2(2):275-285

Hern A, Dorn S (1999). Sexual dimorphism in the olfactory orientation of adult Cydiapomonella in response to alpha farnesene. Entomol. Exp. Appl. 92(1):63-72.

Howcroft KT, Campisi J, Louis GB, Smith MT, Wise B, Wyss-Coray T, Augustine AD, McElhaney JE, Kohanski R, Felipe S (2013). The role of inflammation in age-related disease. Aging 5(1):84-93.

IFRA (International Fragrance Association) (2004). Use Level Survey.

Kobaek-Larsen M, Christensen LP, Vach W, Ritskes-Hoitinga J, Brandt K (2005). Inhibitory Effects of Feeding with Carrots or (-)-Falcarinol on Development of Azoxymethane-Induced Preneo plastic Lesions in the Rat Colon. J. Agric. Food Chem. 53(5):1823-1827.

Lalitharani S, Mohan VR, Regini GS, Kalidass C (2009). GC-MS analysis of ethanoic extract of Pothoss candens L. leaf. J. Herb. Med. Toxicol. 3(2):159-160.

Legault J, Pichette A (2007). Potentiating effect of beta-caryophyllene on anticancer activity of alpha-humulene, isocaryophyllene and paclitaxel. J. Pharmacol. 59(12):1643-1647.

Lopez-Lazaro M (2009). Distribution and biological activities of the flavonoid luteolin. Mini Rev. Med. Chem. 9:31-59.

Magdi MM, Richard EM, Jo-Yun TC, David JA, Samuel WP (2009) Identification and Quantitation of trans-9, trans -12-Octadecadienoic Methyl Ester and Related Compounds in Hydrogenated Soybean Oil and Magarine by Capillary Gas Chromatograhy/Matrix Isolation/ Fourier Transform Infrared Spectroscopy. J. Agric. Food. Chem.

38:86-92.
Mahmoud FMM, Saad AA, Tarek HT, Sulaiman AA (2013). In vitro antifungal activity of Argemon eochroleuca Sweet latex against some pathogenic fungi. Afr. J. Biotechnol. 12(10):1132-1137.
Majno G, Palade GE (1961). Studies on inflammation. 1. The effect of histamine and serotonin on vascular permeability: an electron microscopic study. J. Biophys. Biochem. Cytol. 11:571-605.
Manjunatha BK, Syed M, Divakara R. (2013). Antioxidant and Anti-Inflammatory Potency of Mesua Ferrea Linn. Indian J. Appl. Res. 3(8):55-59.
Masoumian M, Arbakariya A, Syahida A, Maziah M (2011). Flavonoids production in Hydrocotyle bonariensis callus tissues. J. Med. Plant. Res. 5(9):1564-1574.
Noha A, Mohammed IA, Mohamed A, Elfadil EB (2011). Nutritional Evaluation of Sorghum Flour (Sorghum bicolor L. Moench) During Processing of Injera. World Acad. Sci. Eng. Technol. 75:86-112.
Nurettin Y, Canan G, Osman U, Ahmet Y, Serdar U, Kamil C, Salih T (2006). Composition and Antimicrobial Activities of Volatile Components of Minuartia meyeri. Turk. J. Chem. 30:71-76.
Okwu DE, Josiah C (2006). Evaluation of the chemical composition of two Nigerian medicinal plants. Afr. J. Biotechnol. 5:357-361.
Olaniran OA, Sudhakar AVS, Drijfhout FP, Dublon IAN, Hall D R, Hamilton JGC, Kirk WDJ (2013). A male-predominant cuticular hydrocarbon, 7-methyltricosane, is used as a contact pheromone in the western flower Thrips Frankliniella occidentalis. J. Chem. Ecol. 39(4):559-568.
Opie EL (1962) On the relation of necrosis and inflammation to denaturation of proteins. J. Exp. Med. 115:597-608.
Ordonez AAL, Gomez JD, Vattuone MA, Islam MI (2006). Antioxidant activities of Sechiumedule. Food Chem. 97:452-458.
Ouviña A, Gorzalczany S, Acevedo C, Ferraro G. (2009). Topic antiinflammatory activity of extracts from Hydrocotyle bonariensis Lam. (Apiaceae). Lat. Am. J. Pharm. 28(6):941-944.
Paulsen, BS (2010). Highlights through the history of plant medicine. In. Proceedings from a Symposium Held at the Norwegian Academy of Science and Letters, Oslo, Norway.
Rather LJ (1971). Disturbance of function (functio laesa): the legendary fifth cardinal sign of inflammation, added by Galen to the four cardinal signs of Celsus. Bull. N.Y. Acad. Med. 47:303-22.
Reitman S, Frankel S (1957). A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminase. Am. J. Clin. Pathol. 28:56-63.
Roy SP, Niranjan CM, Jyothi TM, Shankrayya MM, Vishawanath KM, Prabhu K, Gouda, VA, and Setty RS (2010). Antiulcer and anti-inflammatory activity of aerial parts Enicostemma littorale Blume. J. Young Pharm. 2(4):369-373.
Ryan GB, Majno G (1977). Acute inflammation. A review. Am. J. Pathol. 86:183-276.
Sadique J, Al-Rqobahs WA, Bughaith EAR (1989). The bioactivity of certain medicinal plants on the stabilization of RB membrane system. Fitoterapia 60:525-532.
Sakat S, Juvekar AR, Gambhire MN (2010). In vitro antioxidant and anti-inflammatory activity of methanol extract of Oxalis corniculata Linn. Int. J. Pharm. Pharm. Sci. 2(1):146-155.
Sangita C, Priyanka C, Protapaditya D, Sanjib B (2012). Evaluation of in vitro anti- inflammatoryactivity of coffee against the denaturation of protein. Asian Pac. J. Trop. Biomed. 2(1):S178-S180.
Shinde UA, Phadke AS, Nari AM, Mungantiwar AA, Dikshit VJ, Saraf MN (1999). Membrane stabilization activity- a possible mechanism of action for the anti-inflammatory activity of Cedrus deodara wood oil. Fitoterapia 70:251-257.
Silva RO, Sousa FB, Damasceno SR, Carvalho NS, Silva VG, Olivera FR, Sousa DP, Aragao KS, Barbosa AL, Freitas RM, Medeiros JV (2013). Phytol, a diterpene alcohol, inhibits the inflammatory response by reducing cytokine production and oxidative stress. Fundam. Clin. Pharmacol. 10:120-149.
Singleton VL, Orthofer R, Lamuela-Raventos RM (1999). Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu Reagent. Methods Enzymol. 299:152-178.
Sivakumar R, Rajesh R, Buddhan S (2007). Antilipidemic effect of chitosan against experimentally induced myocardial infarction in rats. J. Cell Anim. Biol. 1(4):71-77.
Sobotnik J, Hanus R, Kalinova B, Piskorski R, Cvacka, J, Bourguignon T, Roisin Y (2008). (E,E)-α-Farnesene, an Alarm Pheromone of the Termite Prorhinotermes canalifrons. J. Chem. Ecol. 34(4):478-486.
Souza SM, Aquino LC, Milach AC, Bandeira MA, Nobre, M.E, Viana GS (2007). Anti-inflammatory and antiulcer properties of tannins from Myracrodruon urundeuva Allemão (Anacardiaceae) in rodents. Phytother. Res. 21(3):220-225.
Stankov SV (2012). Definition of inflammation, causes of inflammation and anti-inflammatory strategies. Open Inflamm. J. 5:1-9.
Uddin SJ, Grice D, Tiralongo E (2012). Evaluation of cytotoxic activity of patriscabratine,tetracosane and various flavonoids isolated from the Bangladeshi medicinal plant Acrostic. humaureum. Pharmacol. Biol. 50(10):1276-1280.
Umapathy E, Ndebia EJ, Meeme A, Adam B, Menziwa P, and Nkeh-Chungag BN (2010). An experimental evaluation of Albuca setosa aqueous extract on membrane stabilization, protein denaturation and white blood cell migration during acute inflammation. J. Med. Plants Res. 4(9):789-795.
Xing-Jiu H, Yang-Kyu C, Hyung-Soon I, Oktay Y, Euisik Y, Hak-Sung K (2006). Aspartate Aminotransferase (AST/GOT) and Alanine Aminotransferase (ALT/GPT) Detection Techniques. Sensors 6:756-782.
Yang D, Michel L, Chaumont JP, Millet-Clerc J (1999). Use of caryophyllene oxide as an antifungal agent in an in vitro experimental model of onychomycosis. Mycopathologia 148(2):79-82.
Yogeswari S, Ramalakshmi S, Neelavathy R, Muthumary J (2012). Identification and Comparative Studies of Different Volatile Fractions from Monochaetia kansensis by GCMS. Glob. J. Pharmacol. 6(2):65-71.
Yoshida T, Konishi M, Horinaka M, Yasuda T, Goda AE., Taniguchi H, Yano K, Wakada M, Sakai T (2008). Kaempferol sensitizes colon cancer cells to TRAIL induced apoptosis. Biochem. Biophys. Res. Commun. 375:129-133.
Zih-Rou H, Yin-Ku L, Jia-You F (2009). Biological and Pharmacological Activities of Squalene and Related Compounds: Potential Uses in Cosmetic Dermatology. Molecules 14:540-554.

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Full Length Research Paper

Occurrence of *Escherichia coli* in *Brassica rapa* L. *chinensis* irrigated with low quality water in urban areas of Morogoro, Tanzania

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Low quality water has become valuable resource with restricted or unrestricted use in food production depending on its quality. This study has quantified the occurrence of *Escherichia coli* in *Brassica rapa* L. *chinensis* (Chinese cabbage) vegetables and low quality irrigation water. A total of 106 samples including Chinese cabbage (69) and water (37) were collected. The *E. coli* were cultured in petri film selective *E. coli* plates at 44°C. The Chinese cabbage irrigated with river water at Fungafunga area indicated significantly (P<0.001) high prevalence 86% (n=21, 0.00-4.10 log cfu/g) of *E. coli* than those irrigated with treated wastewater at Mazimbu 10% (n=48, 0.00-1.36 log cfu/g). The mean counts of *E. coli* in untreated wastewater ranged from 4.59 to 5.56 log cfu/mL, while in treated wastewater was from 0.54 to 1.05 log cfu/mL and in river water it was 2.40 log cfu/mL. Treated wastewater of the quality found in this study could be used for food production.

Key words: Agricultural irrigation, Chinese cabbage, incidental inputs, river water, wastewater.

INTRODUCTION

The term low quality irrigation water (LQIW) used in this study covers different types of water used for irrigation of crops in urban and peri urban areas of Morogoro municipality. The LQIW include domestic wastewater and polluted downstream rivers. The use of LQIW in food production is considered as an alternative source of water due to physical and economical water scarcity (Mateo-Sagasta et al., 2015). Low quality irrigation water has become valuable resource with restricted or unrestricted use in food production depending on its quality (World Health Organization, 2006a, b, c). It is a valuable and reliable resource for irrigation and fertilizing soils (Babayan et al., 2012) particularly in the Middle East (Ensink et al., 2007) as well as in African countries (Alemayehu et al., 2015). Agricultural irrigation by utilizing wastewater plays a significant role in food security as it improves crop yields and allows year round production (Jiménez, 2006). In Tanzania, for instance in urban and

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peri-urban areas of Morogoro, small-scale farmers around wastewater treatment systems use LQIW (Mayilla et al., 2015) with partially or without any treatment. The growing water scarcity in urban and peri-urban areas in developing countries is among the drivers for farmers to use readily available LQIW (Qadir et al., 2010) which is often cheap or free. The sustainable use and management of LQIW for food production systems may certainly increase crop yields (Valipour, 2013).

Low quality irrigation water is generally contaminated with humans or animals faecal pathogenic microorganisms. The faecal pathogens may cause diseases to farmers, consumers and communities (Abakpa et al., 2013; Cobbina et al., 2013). These studies reported faecal bacterial contamination in vegetables including lettuce and Swiss chards irrigated with LQIW. These vegetables in particular, lettuce are often consumed raw and so they may pose health risks to consumers from faecal bacteria contamination. The green leafy vegetables have been associated with food-borne outbreaks caused by pathogenic bacteria such as *E. coli, Salmonella* spp., *L. monocytogenes* and *Shigella* spp. (EFSA Panel on Biological Hazards (BIOHAZ), 2011). Although Chinese cabbage is heated prior to consumption, inadequate preparation could expose consumers to potential health risks. Although in developing countries, untreated wastewater is often used for irrigation of crops (Jung et al., 2014), data on occurrence of pathogenic bacteria on green leafy vegetables including Chinese cabbage are limited (Erickson, 2010). The aim of this study was, therefore, to determine the extent of contamination of E. coli in Chinese cabbage and low quality irrigation water.

MATERIALS AND METHODS

This study was carried out in November, 2012 at Mazimbu, Mafisa and Mzumbe wastewater treatment systems (Figure 1) as well as at Fungafunga area (Morogoro River). Field visits were conducted to the farms to identify types of vegetables and farming practices. The observation was guided by prepared checklist which was administered to individuals or groups of farmers participated in this study. The Chinese cabbage was chosen from among the identified vegetables in the fields at Mazimbu and Fungafunga area, study sites. The reason for choosing Chinese cabbage was that it takes 3 to 4 months in the field compared to shortlived or once harvested vegetables such as amaranthus and pumkin leaves.

A total of 106 samples including Chinese cabbage (69) and water samples (37) were collected from four study sites; Mazimbu, Mafisa, Mzumbe and Fungafunga area. Out of 69 samples of Chinese cabbage, 48 were collected at Mazimbu and 21 at Fungafunga. Mzumbe and Mafisa study sites were not included because during the period of this study there were no Chinese cabbage being grown. A bundle of three to five leaves from different Chinese cabbage plants were harvested and placed in the sterile polythene bags. A total of 37 samples of water were collected from Mafisa (11), Mzumbe (13), Mazimbu (9) and Fungafunga area (4). Using sterile falcon tubes, about 50 mL of water samples were collected from untreated wastewater inlets and treated wastewater outlets, while at irrigation fields water samples were collected from a hose pipe, canal or intake points and further from treated wastewater downstream. All samples of Chinese cabbage and water were put into a cool box with ice cubes and immediately transported to the Pest Management Centre Laboratory, Sokoine University of Agriculture and analysed in the same day.

The Chinese cabbage vegetables were chopped using a sterile blade to make a composite sample of 50 g into sterile plastic bags. A total of 100 mL of 0.025% sodium dedocyl sulfate solution (SDS) was added into the bag containing 50 g of vegetables, then swirled ten times to recover bacteria from the sample. A small hole was cut at a corner of the plastic bag by a sterile scissor and about 50 mL of homogenate was transfered into sterile falcon tubes and stored at 4 to 8°C until analysis.

Enumeration of *E. coli* was done on 3 M petri film Select *E. coli* (SEC) plates as per 3 M Microbiology Products protocol (St. Paul, USA). The undiluted Chinese cabbage homogenate and water were serially diluted from 1/10; 1/100; 1/1000, continued as required. About 1 mL of the sample from selected dilutions was inoculated on SEC plates and incubated at 44°C for 24 h. Following incubation, all typical blue colonies of *E. coli* regardless of their size and colour were counted and calculated against the dilution factor and reported in cfu/g or mL for Chinese cabbage and water respectively.

The mean counts of *E. coli* in Chinese cabbage and water samples were compared between study sites by Student's *t*-test using SPSS statistics 20.0 of 2011 (IBM, California, USA). The differences of counts of *E. coli* in Chinese cabbage and water between the study sites were reported at $P<0.05$ (Kamoutsis et al., 2012).

RESULTS AND DISCUSSION

Different types of vegetables are grown in the study sites including Sweet potato, Chinese cabbage (*Brassica rapa* L. c*hinensis*), pumpkin leaves, Swiss chard, *Brassica carinata*, amaranthus and cowpeas. Others were okra, tomatoes, eggplant, African eggplant and paddy. The Chinese cabbage was selected for this study. Table 1 shows the irrigation methods, farm pre-harvest practices and possible sources of contamination on Chinese cabbage in different steps. Main types of irrigation methods observed were surface flooding and furrow aided by pumping and or conveyed by gravity. Various types of composite manures, and fertilizers used include poultry manure, tobacco leafy stalks and dust as well as industrial fertilizers such as Calcium, Ammonia and Nitrogen (CAN), UREA and Sulfate of Ammonia (SA), respectively.

This study found high concentration of *E. coli* (4.00 log cfu/g) in Chinese cabbage at the farm level. The potential sources of pathogen contamination during pre-harvest practices may include dust, soil, polluted irrigation water and rodents (Jung et al., 2014). Previous studies conducted on leafy vegetables in Ghana (Keraita et al., 2007) and in Pakistan (Ensink et al., 2007) during pre- and post-harvest handling practices reported faecal pathogens contamination. This may be caused by unhygienic practices during applying the incidental inputs and handling of harvested vegetables at the farms as habitually are placed in contact with soil and often washed with LQIW. There is also a possibility of growth of faecal pathogens during post-harvest handling, transportation and overnight storage at home, and

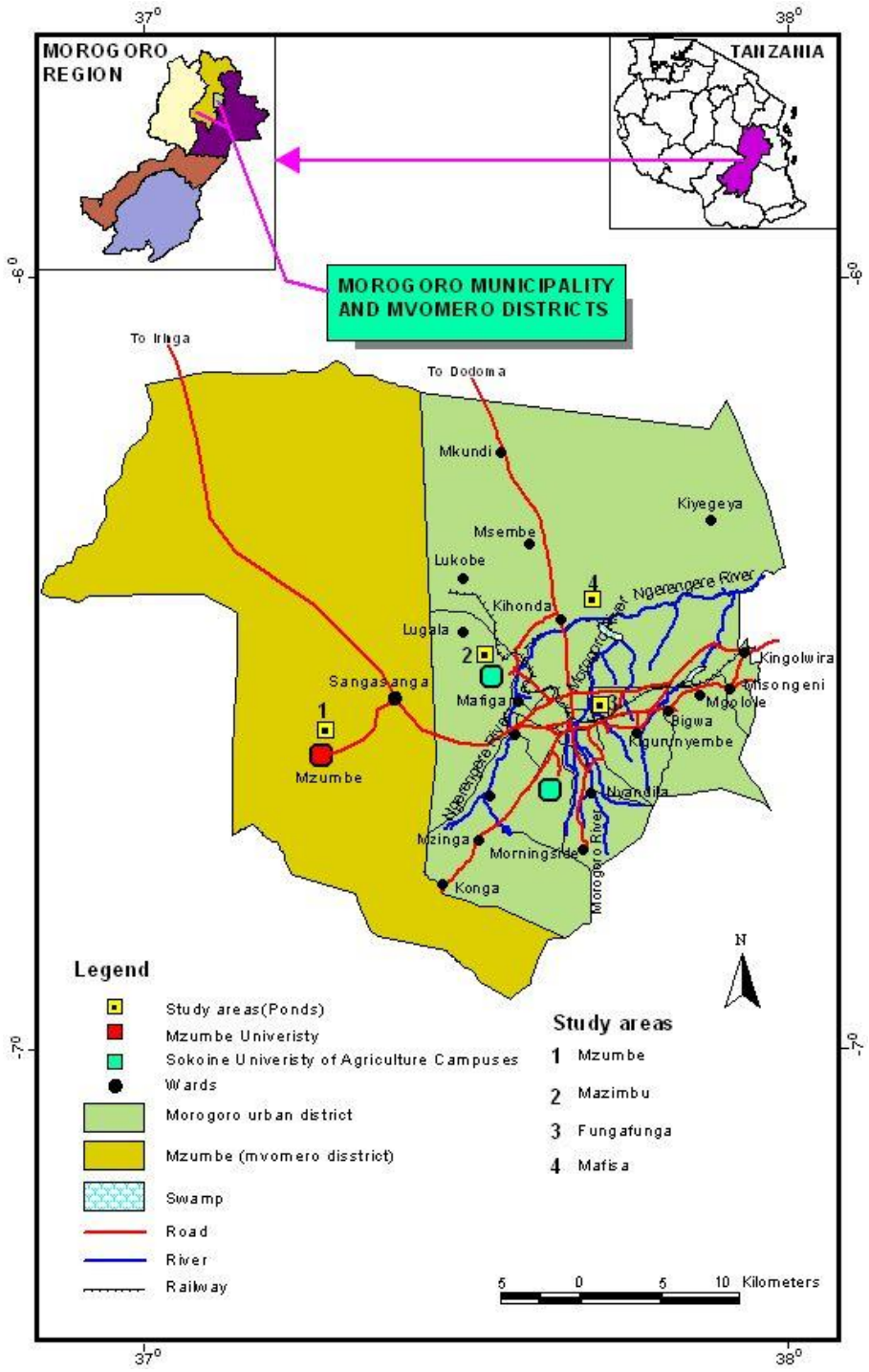

Figure 1. Map of Morogoro urban and peri-urban areas showing study sites (Source: Field Data and Tanzania Administrative Boundaries Map (2002))

display at markets.

The Chinese cabbage irrigated with river water at Fungafunga area indicated significantly (P<0.001) high prevalence 86% (n=21, 0.00-4.1 log cfu/g) of *E. coli* than those irrigated with treated wastewater at Mazimbu 10% (n=48, 0.00-1.36 log cfu/g) (Figure 2). Although there is no established criteria for concentration of *E. coli* in the raw leafy vegetables (ICMSF, 1986), concentration of *E. coli* >3 log cfu/g observed in this study may pose health risks to consumers. This was expected, because

Table 1. Farm practices and possible faecal contamination in Chinese cabbage in urban areas of Morogoro, Tanzania.

Irrigation methods	Steps	Farm practices	Possible contamination/growth of bacteria
Chinese cabbage farms irrigated by flooding using hose pipe, in interval of 2 to 5 days depending on the weather. And or two days prior each harvest.	Field preparation	Fields prepared with or without applying cow or poultry manure, or tobacco stalks	Soil contamination by faecal pathogens from manure, water and incidental inputs
	Seedling preparation and transplanting	Seedlings were planted and left to grow up to the maximum of 1 month and transplanted into the main field	Seedling cross contamination from farmers, water and incidental inputs
	Direct seeding	Some farmers saw seed direct into field by spreading them randomly.	Soil contamination by water and incidental inputs
	Weeding	Weeding depend on growth of weeds.	Contamination with pathogens from soil and incidental inputs
	Fertilizing	Fertilizers were applied during the 3rd week. Most of farmers use poultry manure and sometimes industrial fertilizers (Calcium, Ammonia and Nitrogen – (CAN), UREA and Sulfate of Ammonia (SA)	Possible contamination from poultry manure
	First leafy harvest	First leafy harvest was after 4-6 weeks depending on the early or late maturity	Cross contamination from farmers, handlers and incidental inputs
	Harvesting	Only the mature leaves are harvested every week up to the period of 12 to 16 weeks	Contamination from farmers, handlers and incidental inputs
	Preparation/ consumption	Proper cooking	Reduce or eliminate faecal pathogens load to an acceptable level

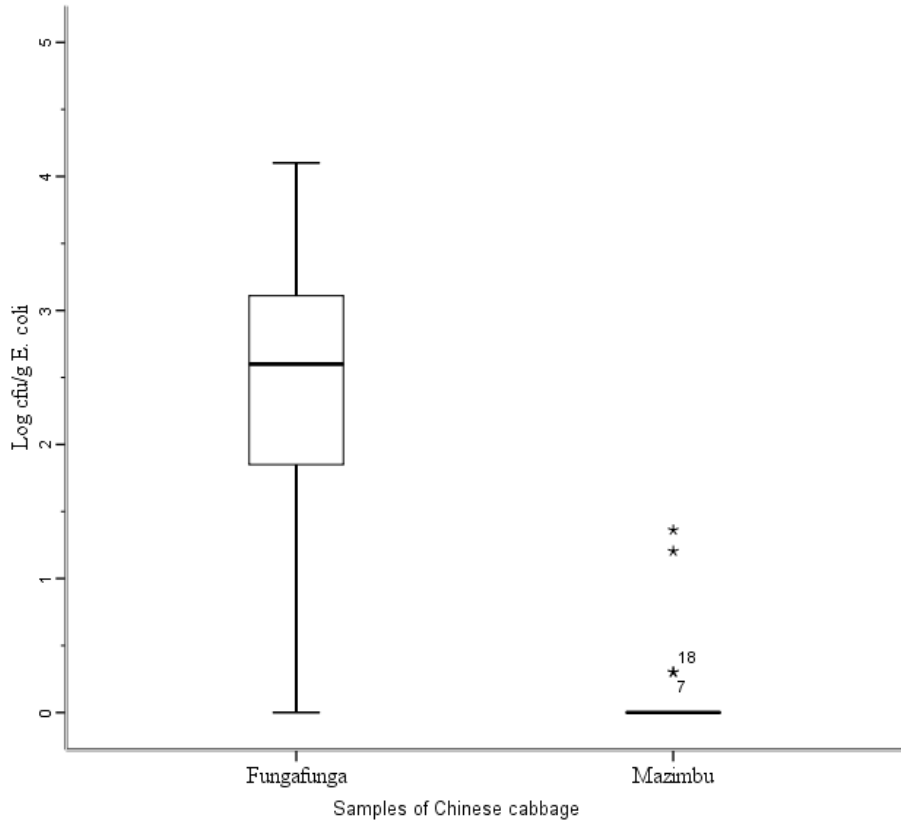

Figure 2. Concentration of *E. coli* in Chinese cabbage vegetables from Mazimbu and Fungafunga. *Concentration of *E. coli* >0 log cfu/g.

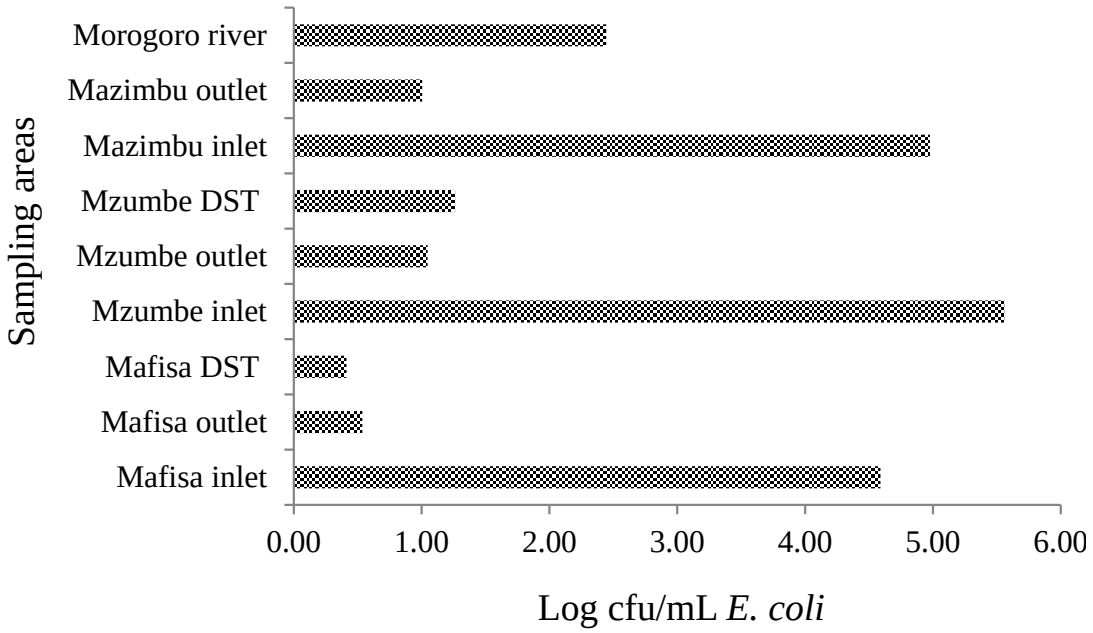

Figure 3. Concentration of *E. coli* in different sources of low quality irrigation water in urban and peri-urban areas of Morogoro (DST, downstream treated wastewater).

Fungafunga area is a home of the old people, located in urban Morogoro. It is, therefore, characterized with high human traffic and activities. Since Morogoro river is not stationary, and if the up-stream experience high domestic activities of effluent of wastewater high contamination levels are expected. The other sources of contamination could be incidental agricultural inputs including animal manure or non-incidental inputs from animals, humans and farmers practices than the irrigation water (FAO/WHO [Food and Agriculture Organization of the United Nations/World Health Organization], 2008; Jung et al., 2014). However, Chinese cabbage which has a natural epiphytic flora, it may acquire contamination from various incidental and accidental inputs.

Figure 3 illustrates the level of *E. coli* contamination of different types of LQIW. The *E. coli* contamination level of untreated wastewater was significantly ($P<0.05$) higher (4.59-5.56 log cfu/mL) than treated wastewater effluent (0.54-1.05 Log cfu/mL) and river water (2.4 log cfu/mL). The untreated wastewater from Mzumbe had the highest level of *E. coli* contamination (5.56 log cfu/mL), while sample from Mafisa was the least (4.59 log cfu/mL) (Figure 2). Likewise, treated wastewater downstream from Mzumbe had highest level of *E. coli* contamination (1.26 log cfu/mL) than the one from Mafisa (0.41 log cfu/mL). If wastewater is not collected from the source and discharged into the wastewater treatment units, it is likely to be disposed indirectly and or directly to the surface water bodies or soil (SAI Platform, 2010). The increase in concentration of *E. coli* in treated wastewater downstream could be due to cross contamination from incidental inputs including environment, human activities, droppings of birds and animal faeces. This may be attributed to the limited or inadequate plans and wastewater treatment facilities, and it thus, may lead to environmental contamination (Sato et al., 2013).

Risk of leafy vegetables contamination with LQIW has been reported to increase in the order of potable/rain water, deep wells and shallow wells. Followed by the surface water in proximity to animals, human habitation and associated wastes; and untreated or partially treated wastewater (Mateo-Sagasta et al., 2013). Low quality water close to human and animal habitation waste or activities may present potential risk when used for food production. The quality of treated wastewater found in the study sites may be used for crop irrigation as recommended by the WHO guidelines (World Health Organization, 2006b). Safe use of treated wastewater for irrigation depends on awareness, knowledge and hygiene practices by the farmers (Mateo-Sagasta et al., 2013). The use of LQIW with *E. coli* >1.00 log cfu/mL for irrigation of vegetables may pose potential health risks to the public and environment. Training of farmers on safe use of wastewater and good agricultural practices will, therefore, help to reduce the potential public health risks (Keraita and Akatse, 2012). Implementation of good agricultural and hygiene practices, good pre- and post-harvest handling practices, may reduce faecal bacterial contamination in irrigated foodstuffs. To our knowledge this is a first study on occurrence of *E. coli* in Chinese cabbage irrigated with treated wastewater in Tanzania. Generally water from rivers is regarded as of good microbiological quality than treated wastewater, and vegetables irrigated by treated wastewater are perceived

of poor quality. Well treated wastewater could be used for crop irrigation with minimal health risks. However, further studies on contamination of pathogenic bacteria in other green leafy vegetables, during pre-and post-harvest handling practices is recommended. Use of tobacco stalks and dust as organic fertilizers in vegetables need to be investigated for their antimicrobial effects.

Conflicts of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

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REFERENCES

Abakpa GO, Umoh VJ, Ameh JB, Yakubu SE (2013). Microbial quality of irrigation water and irrigated vegetables in Kano State, Nigeria. Int. Food Res. J. 20:2933-2938.

Alemayehu S, Tomass Z, Dejene T, Kiros S, Mouz M, Equar G (2015). Physicochemical Quality of Pre-Harvest Vegetables Irrigated with Urban Wastewater in Mekelle and Southern Zones of Tigray Region, Ethiopia. Appl. J. Hyg. 4:18-24.

Babayan M, Javaheri M, Tavassoli A, Esmaeilian Y (2012). Effects of using wastewater in agricultural production. Afr. J. Microbiol. Res. 6:1-6.

Cobbina SJ, Kotochi MC, Korese JK, Akrong MO (2013). Microbial Contamination in Vegetables at the Farm Gate Due to Irrigation with Wastewater in the Tamale Metropolis of Northern Ghana. J. Environ. Prot. 4:676-682.

EFSA Panel on Biological Hazards (BIOHAZ) (2011). Scientific Opinion on the public health risks of bacterial strains producing extended-spectrum β -lactamases and / or AmpC β -lactamases in food and food-producing animals. EFSA J. 9(8):2322.

Ensink JHJ, Mahmood T, Dalsgaard A (2007). Wastewater-irrigated vegetables: Market handling versus irrigation water quality. Trop. Med. Int. Health 12:2-7.

Erickson MC (2010). Microbial Risks Associated with Cabbage, Carrots, Celery, Onions and Deli Salads Made with These Produce Items. Compr. Rev. Food Sci. Food Saf. 9:602-619.

FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization) (2008). Microbiological hazards in fresh leafy vegetables and herbs: Meeting Report. Microbiological Risk Assessment Series No. 14. Rome.

ICMSF (1986). Sampling for Microbiological Analysis: Principles and Specific Applications, In: Micro-Organisms in Foods 2. International Commission on Microbiological Specifications for Foods. Blackwell Scientific Publications.

Jiménez B (2006). Irrigation in Developing Countries Using Wastewater. Int. Rev. Environ. Strateg. 6:229-250.

Jung Y, Jang H, Matthews KR (2014). Effect of the food production chain from farm practices to vegetable processing on outbreak incidence. Microb. Biotechnol. 7:517-527.

Kamoutsis A, Matsoukis A, Kouzounas P, Chronopoulou-Sereli A (2012). Phenological Response of Pear and Orange Trees to Air Temperature at Regions of Thessaly and Peloponnesus, Greece, In. Costas G. Helmis, P.T. Nastos (Ed.), Advances in Meteorology, Climatology and Atmospheric Physics. Springer, Verlag Berlin Heidelberg.

Keraita B, Akatse J (2012). Five easy ways to reduce health risks, In: On- Farm Practices for the Safe Use of Wastwater in Urban and Peri-Urban Horticulture. A Training Handbook for Farmer Field Schools. Food and Agriculture Organization. Food and Agriculture Organization, Rome.

Keraita B, Konradsen F, Drechsel P, Abaidoo RC (2007). Effect of low-cost irrigation methods on microbial contamination of lettuce irrigated with untreated wastewater. Trop. Med. Int. Health 12(Suppl. 2):15-22.

Mateo-Sagasta J, Medlicott K, Qadir M, Raschid-Sally L, Drechsel P, Liebe J (2013). Proceedings of the UN-Water project on the Safe Use of Wastewater in Agriculture, In. Liebe, J., Ardakanian, R. (Ed.), UN-Water Decade Programme on Capacity Development (UNW-DPC).Proceedings Series No. 11. UNW-DPC, Bonn, Germany.

Mateo-Sagasta J, Raschid-sally L, Thebo A (2015). Global Wastewater and Sludge Production, Treatment and Use, In. Drechsel, P., Qadir M., Wichelns, D. (Ed.), Wastewater-Economic Asset in an Urbanizing World. Springer New York London.

Mayilla W, Keraita B, Ngowi H, Konradsen F, Magayane F (2015). Perceptions of using low-quality irrigation water in vegetable production in Morogoro, Tanzania. Environ. Dev. Sustain. pp.1-9.

Qadir M, Wichelns D, Raschid-Sally L, McCornick PG, Drechsel P, Bahri A, Minhas PS (2010). The challenges of wastewater irrigation in developing countries. Agric. Water Manage. 97:561-568.

SAI Platform (2010). Water conservation technical briefs. TB7 – Wastewater use in agriculture. Sustainable Agriculture Initiative (SAI). Brussels, Belgium.

Sato T, Qadir M, Yamamoto S, Endo T, Zahoor A (2013). Global, regional, and country level need for data on wastewater generation, treatment, and use. Agric. Water Manage. 130:1-13.

Valipour M (2013). Evolution of irrigation-equipped areas as share of cultivated areas. Irrigat. Drainage Syst. Eng. 2:e114.

World Health Organization (2006a). WHO Guidelines for the Safe Use of Wastewater, Excreta and Greywater: Volume I - Policy and Regulatory Aspects. World Health Organization, Geneva, Switzerland.

World Health Organization (2006b). WHO Guidelines for the Safe Use of Wastewater, Excreta and Greywater: Volume III - Wastewater and Excreta Use in Aquaculture. Geneva, Switzerland.

World Health Organization (2006c). WHO Guidelines for the Safe Use of Wastewater, Excreta and Greywater: Volume IV. Excreta and Greywater Use in Agriculture. Geneva, Switzerland.

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African Journal of Biotechnology

Full Length Research Paper

Application of polymeric nanoparticles for controlled release of ethanolic extract of guapeva leaves (*Pouteria gardneriana* Radlk) against *Riphicephalus* (*Boophilus) microplus* through *in vitro* studies

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The study was designed to evaluate the ethanolic extract acaricide action from *Pouteria gardneriana* Radlk specie adsorbed in chitosan nanoparticles for controlled release against *Riphicephalus* (*Boophilus) microplus.* Leaves of *P. gardneriana* Radlk were collected, air-dried, ground and extracted with ethanol. Chitosan nanospheres were obtained by the phase inversion method. Conductometric titration, UV-Vis and FTIR analysis were conducted with the materials to evaluate the chitosan anchoring ability. *In vitro* test was performed using engorged females for each treatment, which consisted of the control, raw extract and three treatments diluted, with increasing concentrations of 0.2, 0.4 and 1%. The most effectiveness extract was for 0.4% concentration by observing reduction in the eggs mass compared to control group. The nanomaterial proved to be able to anchor and release the insecticide gradually in pH between 5 and 6 regions, which makes it feasible for use in cattle, prolonging the exposure time between the tick and acaricide.

Key words: Extracts, nanoparticle, controlled release, tick.

INTRODUCTION

Tick *Riphicephalus (Boophilus) microplus* is a livestock parasite that is responsible for decrease in production and this has led to losses estimated to 2 billion dollars per year (Junior and Oliveira, 2005). Besides tick parasitism affect meat and milk quality through inoculation of toxins to the host, it can transmit viral and bacterial diseases and protozoan infections (Massard and Fonseca, 2004). The chemical acaricides is the

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principal tool for tick control. In Brazil there are 6 principal acaricidal classes, but improper use of them has generated resistant strains. It can be explained by the selection and recombination of resistance genes in tick populations exposed to acaricides selection pressures (Hocayen and Pimenta, 2013).

Consumer demand for meat and milk free of chemical residues and the search for new molecules that present adequate toxicity for tick control are reasons for increased interest in plant species that have bioactive potential. In this context plant extracts of some species of Brazilian cerrado are targets for research (Rocha et al., 2011).

Guapeva is a large tree belonging to the family Sapotaceae and genus *Pouteria*. The most abundant secondary metabolites in this species are phenolic compounds, especially tannins (Rocha et al., 2011). The tannins present toxicological activity for some insect species, making guapeva interesting for studying and verification if its leaves extract is effective in *R. (B.)microplus* control (Monteiro et al., 2005; Silva et al., 2009).

Botanical acaricides have many advantages, they have low toxicity, their source is renewable depending of specie, besides being considered biodegradable (Agnolin et al., 2014). It is possible to prolong the action of the herbal products by using a material that can adsorb and release them in a controlled way. In this context the use of polymeric materials that have the capacity mentioned above can increase the product effectiveness (Saha and Ray, 2013).

Chitosan is a polymer material obtained from chitin deacetylation reaction, the second most abundant natural polymer in nature. This polymer has biochemical and physicochemical properties, considered biodegradable, biocompatible, non-toxic besides being a low-cost product. It presents various applications such as adsorption of ions, in the pharmaceutical and food industries, for functionalized precursor molecules, removal of heavy metals and dyes tributaries, among other applications (Witt et al., 2010).

Considering the bioactive potential of some species of Brazilian cerrado and their toxicological activity of tannins group as described by Scalbert (1991).The presence of tannins in the *Pouteria gardneriana* Radlk species makes this plant useful for use in control of *R. (Boophilus) microplus* tick. Hence, the present study aimed at verifying the efficacy of the leaves ethanol extract along with the controlled release technology by the chitosan nanoparticle surface.

MATERIALS AND METHODS

Collection and preparation of plant material

Plant leaves collection

The *Pouteria gardneriana* Radlk Guapeva leaves were collected from Goiano Federal Institute (Instituto Federal Goiano) on the Rio Verde campus, Brazil (17º 48' 16" S, 50º 54' 19" W, 749 m altitude). After collecting them, they were separated from their stems and weighed. The collected plant materials were dried in an oven with forced air (CIENLAB, CE-220) at a temperature of 40°C and ground to fine powder by use of an electrical grinder (SPLABOR, SP-31) to facilitate the extraction.

Extraction

The fine ground powder of *P. gardneriana* Radlk leaves were weighed, extracted with ethanol and residues were filtered using Whatman filter paper no 2. The extract was concentrated using rotary evaporator (LABORGAS, LGI-52CS-1), obtaining the DCE (dried crude extract).

Nanospheres production

The chitosan solution was prepared with 5% (v/v) acetic acid. The mixture formed was under stirring until complete homogenization was achieved. Chitosan solution was introduced into a "spray dryer" system in which the sample passed through a nebulizer to form an aerosol. Other solution was prepared, NaOH at 10% in 100 ml of distilled water. The solution was standardized until getting a 0.995 correction factor. The aerosol dropped down in the NaOH solution mentioned above, which aerosol was gelled in the chitosan nanospheres form. The nanospheres, obtained were washed until reaching pH 7. Subsequently the nanospheres were exposed at room temperature for drying. All this method is known as phase inversion (Dias et al., 2008).

Anchoring and conductimetric titration

For raw extract anchoring on the chitosan nanosphere surface, 1 g of nanosphere and 0.5 g of raw extract were used. It was prepared 5% (v / v) hydrochloric acid (Sigma Aldrich) solution in 10 ml. 60 mL of distilled water in the solution was added. The solution was stirred for 24 h.

The conductivity and pH meter was used to check the solution conductivity and pH before and during the titration. The titration started at pH 2, then added gradually amounts (µL) of NaOH in the system. As pH and conductivity suffered variations by changing their values, aliquots were removed of each variation in conductivity for subsequent analysis in the UV-vis and FTIR. All procedures were performed in triplicate.

Fourier transform infrared (FTIR)

Analyses were performed by FTIR-ATR-NIRA- Frontier PerkinElmer simultaneous with the collection of aliquots during titration. It was observed the relationship between transmittance and wavelength in the spectra. For comparative purposes, were analyzed as aliquots as extract and chitosan solution at 5% and was observed and the shift of the energy band.

Ultraviolet in the visible region (UV-Vis)

During the conductimetric titrations, the collected aliquots at the points of greatest conductivity variation were analyzed separately in the UV-Vis Lambda 750 PerkinElmer, which did full scan spectrum (200 to 1000 nm) to obtain the samples gradients absorbance. For this analysis, we used 200 µl of each sample diluted in 4 ml of ethanol.

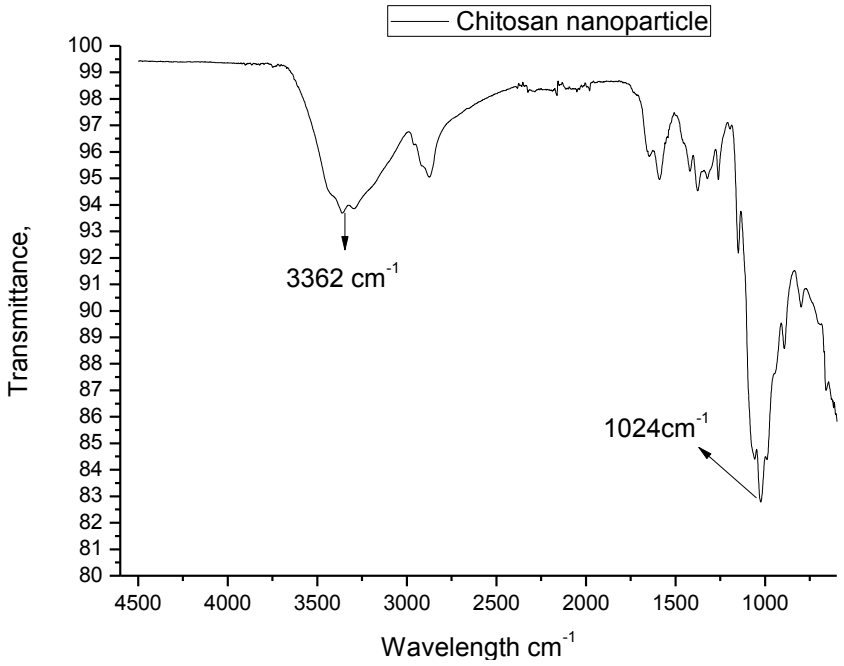

Figure 1. Chitosan nanoparticle spectrum in the FTIR, showing the principal bands and deformation peaks.

Analyses by scanning electron microscopy (SEM)

Nanoparticles were analyzed by Scanning Electron Microscope (SEM), JEOL JSM - 6610, equipped with EDS, Thermo scientific NSS Spectral Imaging, using metallizer BALTEC SCD 050 for nanoparticles morphological determination, as described by Costa et al. (2006).

***In vitro* test**

The *in vitro* test was performed according to Drummond et al. (1973). *R. (B.) microplus* engorged females were collected in naturally infested cattle from afarm near Rio Verde city, Goiás, Brazil. In the laboratory, they were washed with distillated water, dried and separated into 5 groups. The study consisted of five treatments, group I was control group (ethanol), group II corresponded to the raw extract and the groups III until V were the groups in which the raw extract was diluted in ethanol at concentrations of 0.2, 0.4 and 1%, respectively. In treatments III, IV and V were added 0.5 g of chitosan nanosphere and solutions were stirred for 24 h before the start of the tests.

The engorged female ticks were immersed in 20 ml of the solutions corresponding to each treatment for 5 min; this time was established by Leite (1988). After immersion, the engorged females were dried on paper towels and fixed by adhesive on Petri plates, previously identified. 6 engorged females for all groups were performed in triplicate. Next, these were taken to BOD incubator (28 ± 1°C, 80% humidity) for 14 days. After the laying period, the eggs of each tick female for all treatments were transferred to syringes without tips, sealed with cotton, and again sent to the BOD incubator (28 ± 1°C, 80% humidity) where they were kept for 26 days to evaluate for egg hatchability. The treatments were performed in triplicate and the results were obtained from averages. Data were submitted to analysis of variance and means compared by Tukey test at 5% error probability.

RESULTS AND DISCUSSION

The infrared radiation when absorbed by molecules is converted into molecular vibration energy. The spectrum reflects the vibration movement and is usually represented in bands form. The band intensity is measured by transmittance, which is the ratio for the electricity fed into the incident energy. When the molecules suffer changes in their structure, in this case, the adsorption of neighbor molecules by active sites, the new spectra has different characteristics. The bands displacement, increase electron density in the groups, increase the stretch peaks to the emergence of new bands (Aragão and Messaddeq, 2010).

Figures 1 and 2 show the nanoparticle, chitosan and guapeva extract spectra. It is observed bands and stretch peaks for chitosan in the 3362 and 1024 cm^{-1} regions, such regions correspond to angular deformation of N-H, O-H bond and C-O stretching. In Figure 2, bands and deformation peaks in the 3338 and 1046 cm^{-1} regions are observed, including some organic functions, for example, aromatic groups, alcohols, phenols, alkylphenols and carboxylic acid. Those regions found in Figure 2 can be explained by *P. gardneriana* Radlk's secondary metabolites, tannins, which there are aromatic rings with their substituents, especially hydroxyl.

In comparison with the figures mentioned, Figure 3 shows the extract spectrum anchored in the chitosan nanoparticle. The bands in the 3362 and 3338 cm^{-1} regions of the chitosan and the extract, respectively, suffered displacement, as evidenced by the new band

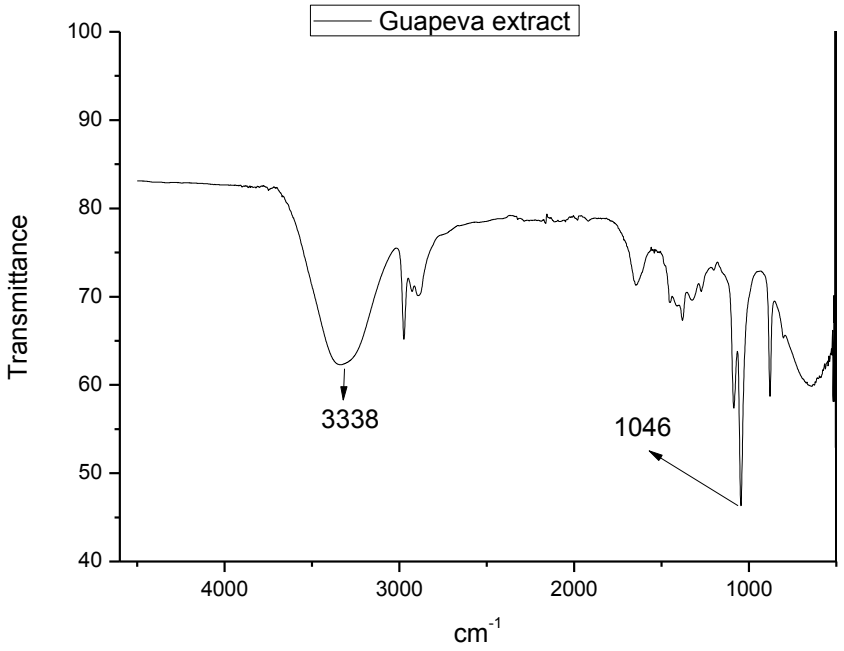

Figure 2. Guapeva extract spectrum in the FTIR, showing the principal bands and deformation peaks.

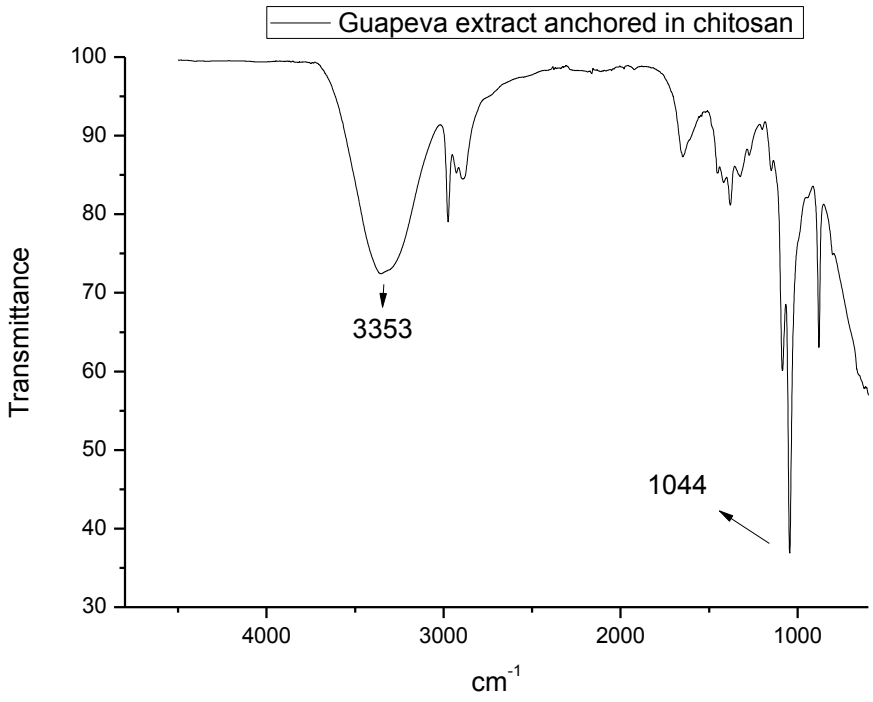

Figure 3. Extract spectrum anchored in chitosan in the FTIR, showing the principals bands and deformation peaks.

appearance in the 3353 cm^{-1} region. Regarding the stretch peak, it is observed in Figure 3 that 1044 cm^{-1} region there was an electron density increase, an increase in stretch peak and consequently a decrease in the transmittance intensity due to the interactions occurring between polymer surface and the molecules

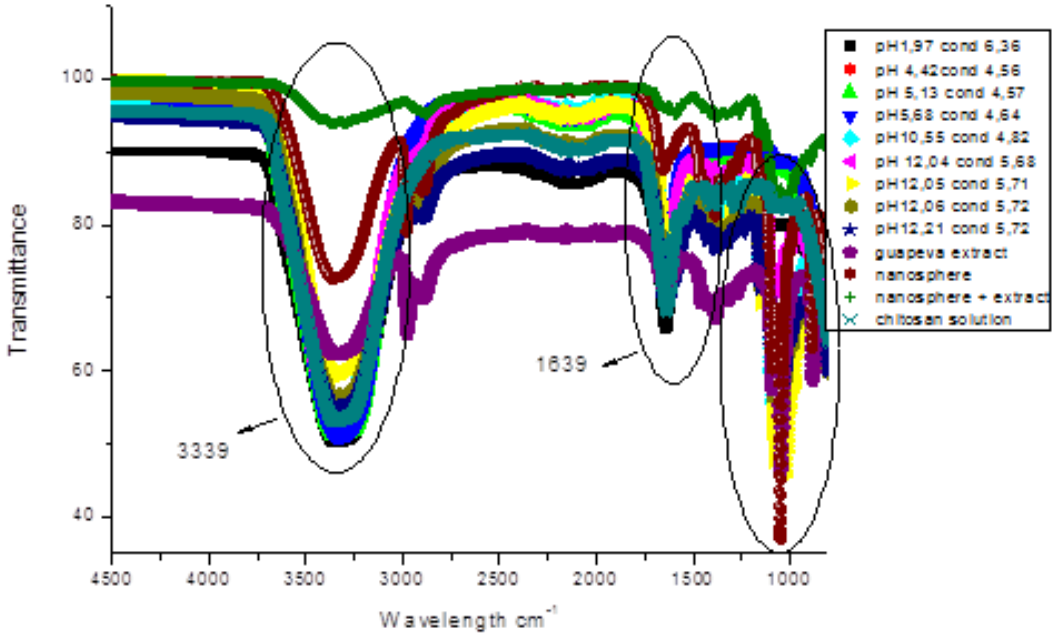

Figure 4. Infrared spectrum of the conductivity variation points obtained during conductimetric titration, the bands displacement according to pH solution.

present in the extract.

In addition, from infrared analyzes with the aliquots collected during conductimetric titration, it is clear that the infrared bands are displaced. Characteristic peaks in the 3500, 1600 and 1000 regions suffered increase and decrease in the transmittance intensity due to the interactions occurring on the polymer surface and the extract molecules, as shown in Figure 4. A higher electron density for spectra that were closer to neutral pH was observed, for example at pH 5.13 and 5.68. The electron density is linked to organic compound release by the polymer matrix. The FTIR analysis justify the appearance of other peaks found in the UV-Vis analyzes, because of interactions occurring during the release process promoting the formation of different spectra of those initial before conductmetric titration process.

Studies performed by Baroni et al. (2007) showed the same behavior of chitosan infrared spectra modified with glutaraldehyde and epichlorohydrin reagents adsorbing chromium ions. The authors demonstrated changes in the spectrum after ions adsorption, concluding that the ions adsorption affects amino group bonds, and the nitrogen atoms are the main adsorption sites for the ions (Baroni et al., 2007).

During the titration process, the solution conductivity changed according to the volume of NaOH added. For greater changes, aliquots were collected and analyzed in the UV-Vis. Depending on the pH, the behavior of the spectra analyzed took another form. The appearance of peaks is shown in Figure 5, besides an intensity increase in the UV absorbance bands according to the pH of the solution. From the figure, it can be observed that the absorbance peaks reached 0.80 and 0.79 at 331 cm^{-1} for pH 2.07 and 2.08, respectively. This shows that in acidic medium chitosan is more protonated and through the largest absorbance peaks observed in the graph, it is stated that the adsorbed extract concentration is higher. The amino group protonation and deprotonation by the chitosan molecule are responsible for solution electronic conductivity. Moreover, higher or lower extract concentrations in the medium are shown by the conductivity difference.

In contrast, there was a peak intensity decrease at 300 cm^{-1}, when the solution was in pH 6.70 and 7.95, while the absorbance values found were 0.50 and 0.47, respectively. Another noted peak is in pH 6.25, at this point there is an increase in absorbance peak, so inferring for the solution ionic equilibrium. There was higher extract concentration released for the medium at this pH. As the solution becomes more alkaline, the solution tendency is occurring deprotonation of the amine group, thereby releasing the adsorbed material. Higher solution ion exchange is, higher will be the solution conductivity, it naturally occurs by neutralization reaction with acid-base, but with the extract adsorption by the polymer surface, the medium conductivity increases and decreases, as a result of adsorption and release effects of the active ingredient.

The relationship between conductivity and pH is demonstrated as shown in Figure 6. The solution

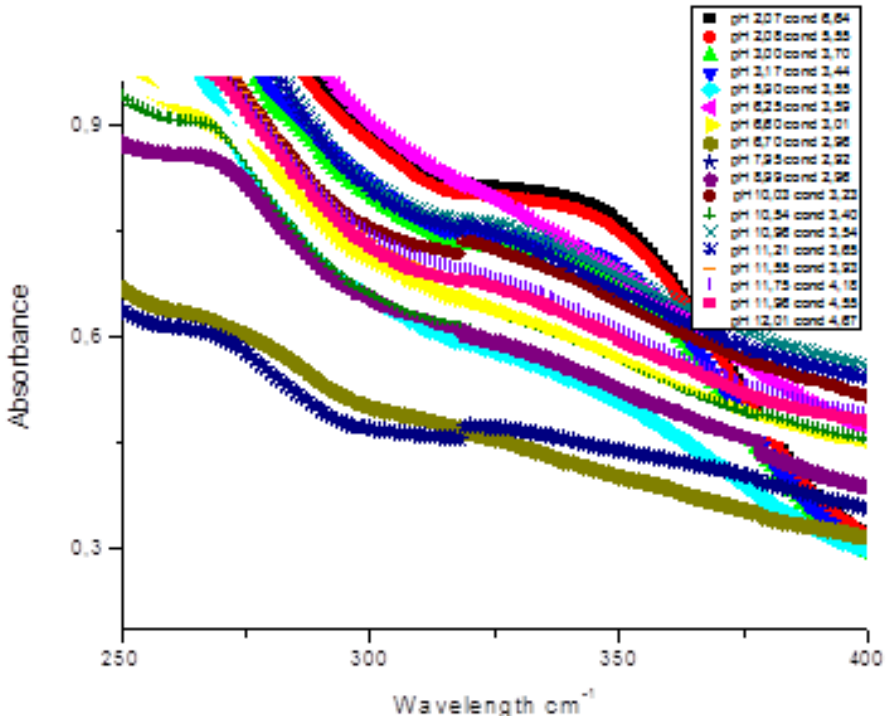

Figure 5. UV-Vis spectrums represented by conductometric titration curves, shown the peaks intensity increase for same spectrums close to neutral pH.

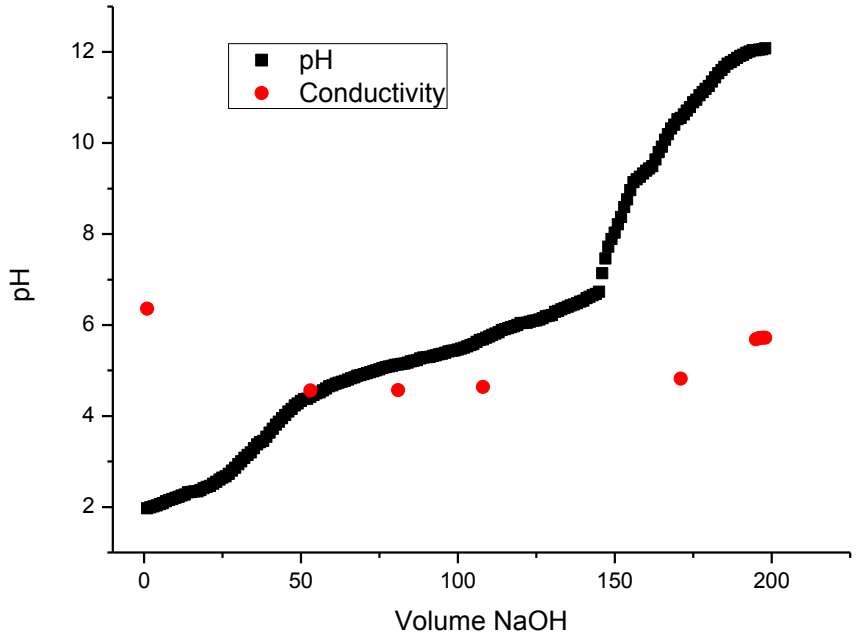

Figure 6. Solution pH and conductivity during the conductimetric titration.

conductivity decreases due to the fact that H^+ from the solution still in acid pH, is being consumed by the OH^- base, that is being added, but the pH ranged between 4.5 and 5, there was a significant increase in conductivity, which could be explained by the extract molecule release from the biopolymer surface.

The images taken by SEM shows that the methodology used to produce nanoparticles was efficient, displaying

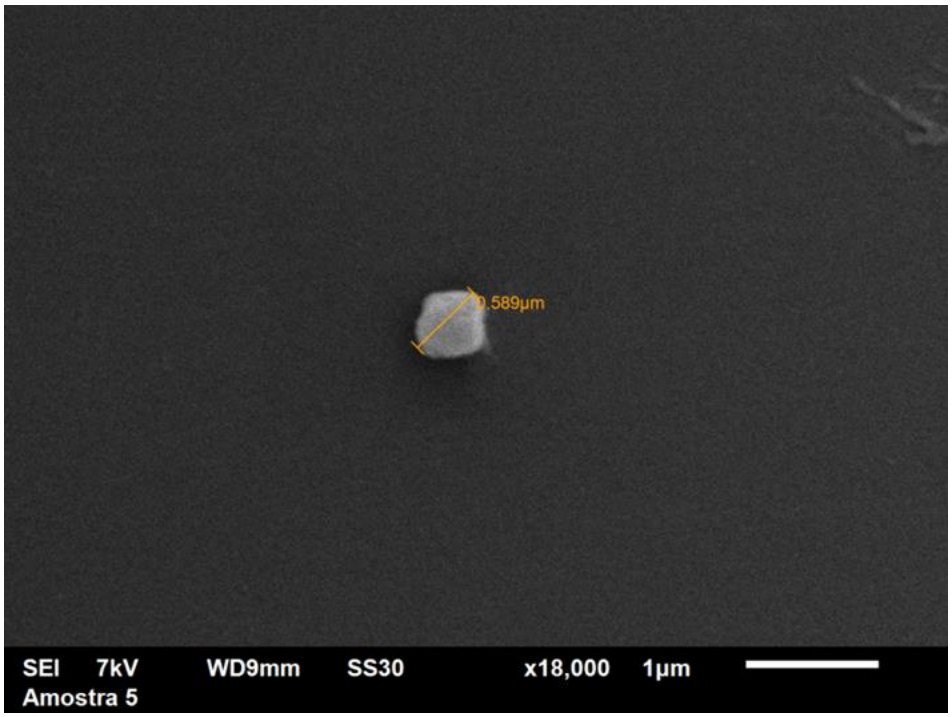

Figure 7. SEM image of nanoparticles produced from spray-dry system by gelation method in NaOH.

uniformity in particle size, about 589 nm, as shown in Figure 7.

The extracts toxicity determination associated with controlled release performed by the nanoparticles was determined by counting dead ticks in different concentrations of the extract anchored in the nanoparticles.

It was observed that for the raw extract treatment, 100% of extract concentration, occurred 100% of death for all individuals, so in higher concentration, the *P. gardneriana* Radlk extract acts as an effective acaricide.

After 14 days from beginning of bioassay, the ovipostion eggs were collected and weighed separately. Eggs mass reduction is associated with the concentration used for treatments preparation. The graph below (Figure 8) relates the eggs mass reduction by the treatments used, using Except for treatment 2 (raw extract), the treatment IV, 0.4%, had the highest percentage of eggs mass reduction. Treatments A, B and C correspond to same the treatments, but done three times. Different treatments are expressed by roman letters.

Figure 9 represented by the control, the raw extract and 0.4% concentration treatments oviposition, numbered 1, 2 and 3, respectively. It is notable the eggs mass reduction in the treatment 4 compared with the control, this confirms the results of statistical tests performed, in which pointed to extract effectiveness. In addition, death for all individuals is represented in Figure 9-2, it is understood that at this concentration the extract active molecules completely inhibit the parasite's cycle; however the use of this becomes unfeasible due to the amount of material used in its production.

Through the obtained results, it is possible to determine that the extract concentration of 0.4%, the eggs mass reduction, together with the number of deaths and non oviposition was significant at 5% Tukey test, which proves the efficiency of controlled release technique of *P. gardneriana* Radlk extract.

Conclusion

The spray-drying methodology is efficient in the nanoparticles production. The chitosan applicability as an anchoring material and in the controlled release study was effective. There was higher release extract between pH 5 and 6, promoting the active ingredient slow release, increasing the exposure time between the tick and acaricide. When raw extract treatment (treatment II) was used, all individuals died before spawning occur. For diluted treatments, as the concentration increased the egg mass decreased, thus the concentration (treatment IV) of 0.4%, the results were considered significant at the 5% Tukey test.

Conflicts of Interests

The authors have not declared any conflict of interests.

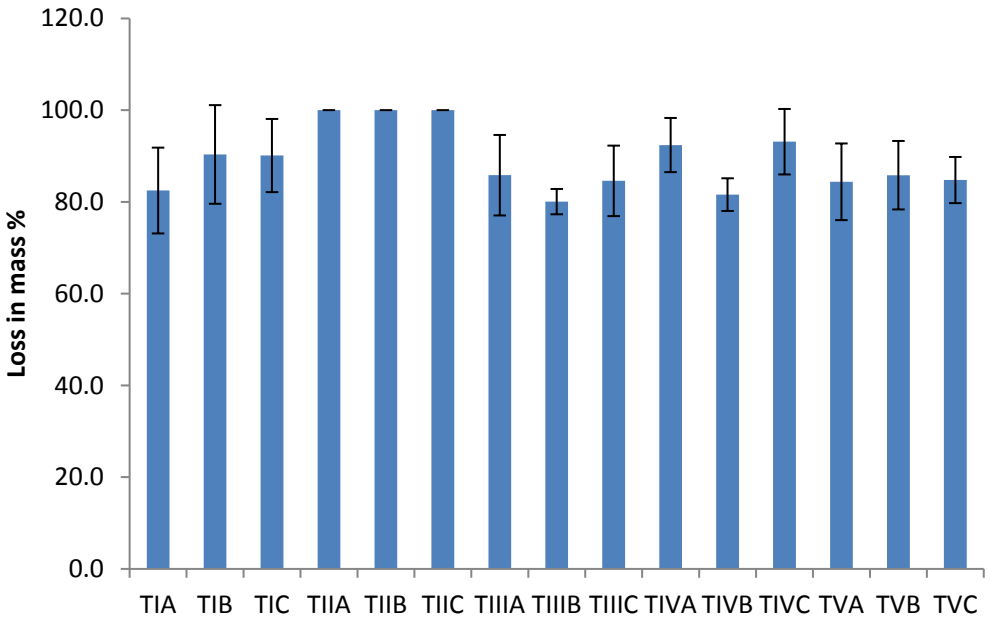

Figure 8. Reduced rate representation in percent of the eggs mass after 14 days from the beginning the treatment.

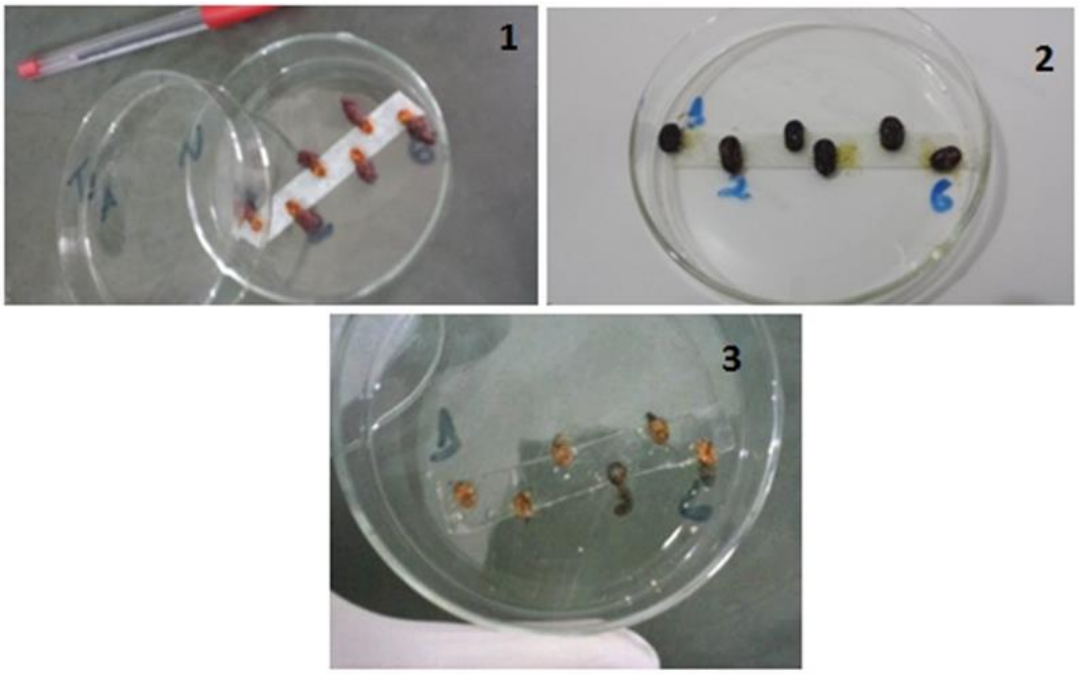

Figure 9. Control treatment, raw extract and concentration of 0.4% oviposition after 14 days from the beginning of the bioassay.

REFERENCES

Agnolin, CA, Olivo CJ, Parra CLC (2014). Efeito do óleo de capim limão (*Cymbopogon flexuosus* Stapf) no controle do carrapato dos bovinos. Rev. Bras. Plantas Med. 16(1):77-82.

Aragão BJG, Messaddeq Y (2010). Peak separation by Derivative Spectroscopy Applied to FTIR Analysis of Hydrolized Silica. J. Braz. Chem. Soc. 19(8):582-1594.

Baroni P, Viera RS, Meneghetti E, Silva MGC, Beppu MM (2007). Evaluation of batch adsorption of chromium ions on natural and

crosslinked chitosan membranes. J. Hazard. Mater. 4:1-9.
Costa ACFM, Vilar, M A., Lira HL, Kiminami RHGA, Gama L (2006). Síntese e caracterização de nanopartículas de TiO_2. Cerâmica 52(324):255-259.
Dias FS, Queiroz DC, Nascimento RF, Lima MB (2008). Um sistema simples para preparação de microesferas de quitosana. Quím. Nova 31(1):160-163.
Drummond RO, Ernst SE, Trevino JL, Gladney WJ, Graham OH (1973). *Boophilus annulatus* and *B. microplus* laboratory tests of insecticides. J. Econ. Entomol. 66(1):130-133.
Hocayen PAS, Pimenta DS (2013). Extrato de plantas medicinais como carrapaticida de *Rhipicephalus (Boophilus) microplus*. Rev. Bras. Plantas Med. 5(4):627-631.
Junior DAC, Oliveira PR (2005). Avaliação *in vitro* da eficácia de acaricidas sobre *Boophilus microplus* (Canestrini, 1887) (Acari: Ixodidae) de bovinos no município de Ilhéus, Bahia, Brasil. Ciênc. Rural 35(6):1386-1392.
Massard CL, Fonseca AH (2004). Carrapatos e doenças transmitidas comuns ao homem e aos animais. A Hora Veterinária 23:15-23.
Monteiro JR, Albuquerque UP, Araújo EL (2005).Taninos: Uma abordagem da química á ecologia. Quím. Nova. 28(5):892-896.
Rocha WS, Lopes RM, Silva DB, Viera RF, Silva JP, Agnostini-Costa TS (2011). Compostos fenólicos totais e taninos condensados em frutas nativas do cerrado. Rev. Bras. Frutic. 33(4):1215-1221.
Saha AK, Ray SD (2013). Effect of cross-linked biodegradable polymers on sustained release of sodium diclofenac-loaded microspheres. Braz. J. Pharm. Sci. 9(4):873-888.
Scalbert A (1991). Antimicrobial properties of tannis. Phytochemistry 30(12):3875-3883.
Silva Cam, Simeoni La, Silveira D (2009). Genus Pouteria: chemistry and biological activity. Rev. Bras. Farmacogn. 19(2):501-509.
Witt MA, Barra GMO, Bertolino JR, Pires ATN (2010). Crosslinked Chitosan/Poly (Vinyl Alcohol) Blends with Proton Conductivity Characteristic. J. Braz. Chem. Soc. 21(9):1692-1698.

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Full Length Research Paper

Allelopathic effect of aqueous extract of fresh leaf castor beans (*Ricinus communis* L.) applied to the beginning stage of soy (*Glycine max* L.) and safflower (*Carthamus tinctorius* L.)

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Allelopathy defines the production of specific biomolecules (allelochemical) by a plant that can induce positive or negative impacts on another culture. The crop of castor beans (*Ricinus communis* L.) is being economically valued and receiving attention, mainly by the biodiesel production, castor oil and animal feeding. In this study, the allelopathic effect of aqueous extract from fresh leaves of castor beans (*Ricinus communis* L.) applied to the beginning stage of soy (*Glycine max* L.) and of safflower (*Carthamus tinctorius* L.) was investigated. An experimental was setup with five blocks and four replications, considering that each block refers to a concentration of extract of castor beans fresh leaf (0, 25, 50, 75 and 100%); the one without castor beans leaves was taken as 'witness'. The statistic treatment was done using analysis of variance (ANOVA) and Tukey test with an error rate of 5%. The result showed evidences that there are allelopathic effects among the extract of castor beans fresh leaf and the beginning stage of soy and safflower only in the root part of the seedling.

Key words: *Ricinus communis L., Glycine max* L., allelopaty.

INTRODUCTION

Allelopathy term defines the production of specific biomolecules (allelochemicals) by a plant that can induce positive or negative impacts on other cultures (Babula et al., 2009). There are many ways for a plant to release its allelopathic components: By volatilization, by soil through roots and leaves, that after they have fallen, they

decompose and release allelopathic substances (Rizvi et al., 1992).

Safflower (*Carthamus tinctorius* L.) is an oleaginous of multiple purposes and it has been cultivated for many centuries in the worldwide, mainly in Iran, Argentina, Kazakhstan, Mexico and in Brazil in the last years (FAOSTAT, 2013; Silva, 2013). In the ancient times, it was used as pigment and tea, but only in the last century its oil started being used for food and industrial purposes, due to its high level of quality (Ahmed et al., 2007; Sehgal and Raina, 2005). It is rich in unsaturated fatty acids with the linoleic acid proportion of about 78% in relation to the total fatty acids. It has red and orange pigments extracted from its flowers (Davood et al., 2013). In Brazil it has been receiving emphasis as second harvest crop, and in 2013 the Brazilian sunflower and safflower oil exportation reached a participation of 0.05% in the world market (Guidorizzi, 2016; MARA, 2013).

Soy is one of the main responsible for the concept of agribusiness and cultivation of grain. In world level, it represents the main oilseed consumed and produced. Its importance is justified by the fact that it may be used for animal consumption and for human consumption. The soy complex has a chain of processes which are the base for the Brazilian economy, which transforms the raw product in the most diverse uses, as animal feed, oil derived (Furtuoso and Guilhoto, 2013).

According to FAO (2016), soy (*Glycine max* L.) will continue being the crop that produces more oil and protein until 2025, at least, because of its great world demand of products derived of this culture. However the projections show that Brazil will overcome the United States in the production ranking because of its productivity and available area. In the same publishing, FAO states that the increasing demand for grains rich in protein and oil has been the main reason of the expansion of oleaginous production in the past years.

As the biodiesel and castor oil production, the castor beans (*Ricinus communis* L.) cultivation is being economic valued and receiving attention from the producers (Costa et al., 2014). According to CONAB (2016) the estimation is that Brazil will raise its production, planted area and productivity in the next years. According to Santos et al. (2011), another reason of interest for castor beans is the possibility that it is a culture for familiar crop because it is easily adaptable and resistant to the climate and soil conditions.

Recent studies done by Kpanja et al. (2016) concluded that if the castor beans pie is cooked for 30 min on steam it can complement or even substitute the peanut pie to the level of 50% in the diets of broiler chicken, showing new purposes for castor beans products and stimulate the interest for castor beans crop.

This paperwork aims to verify if the aqueous extract of castor beans fresh leaves has influence on soy and safflower germination and development in its beginning stage, when this fluid is applied with certain frequency comparing to seedling that did not receive this treatment.

MATERIALS AND METHODS

Study location

This study was conducted at Universidade Estadual do Oeste do Paraná[State University from the West of Paraná], UNIOESTE, campus from Cascavel, Paraná, Brazil, with geographic coordinates 24° 57' 21"S and 53° 27' 19"W and average height of 781 m above sea level. The seedling in the greenhouse was done in the campus and the analysis at Laboratório de Física dos Solos [Soil Physics Lab]. The experiment with soy was conducted with Nidera 5909 variety and with a safflower genotype made available by the Instituto Agronômico do Paraná [Agronomic Institute from Paraná] (IAPAR).

Experimental design

Fresh castor beans leaves were collected from UNIOESTE campus with the geographic coordinates of 24º 57'21"S and 53º 27'19"W and average altitude of 781 m. The collected materials were triturated with electric blender and sieved for the extract preparation. For the seedling, 10 seeds were disposed in a spread way, each treatment had 4 repetitions, totalizing 40 seeds per treatment. The germinating of seeds occurred in tubes of polyvinyl chloride (PVC) with diameter of 200 mm and height of 15 cm, housed in trays with sufficient diameter heights to accommodate the PVC pipes. The deepness of the seeding was constant in all the treatments, being 1.5 cm.

Preparation of extract

The extract was manufactured in concentrations of 0 (control), 25, 50, 75 and 100% of extract to induce the treatment, considering 200 grams of leaf per each liter of cold water, the concentrations were represented by the terms T1, T2, T3, T4 and T5. For the irrigation, 500 ml of water was used, divided into the concentrations. The extract production process consists in grinding mate leaves along with water and then filtered to remove the particles.

Evaluation of allelopathic effects

Germination evaluations were done daily after the installation of the experiment; it started on the first day and ended on the fifteenth day. It is necessary to consider that the stabilization was reached on the thirteenth day on safflower and twentieth day on soy and we considered a germinated seed the one to show 2 mm of protrusion root (MARA, 2009).

There was a difference in relation to the evaluated germination, which was: First germination counting (PCG) –done on the ninth day after the experiment installation (MARA, 2009).

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Table 1. Tukey trust break of parameters evaluated in soy.

GSI (A)			FGC (B)		
Factors	**Averages**	**Group**	**Factors**	**Averages**	**Group**
T1	0.62(0.58)	a	T1	1.50(1.00)	a
T2	0.51(0.29)	a	T2	1.75(0.96)	a
T3	1.05(0.73)	a	T3	1.75(0.50)	a
T4	0.67(0.33)	a	T4	2.25(0.96)	a
T5	0.67(0.27)	a	T5	2.25(0.50)	a
AGT (C)			**G (D)**		
Factors	**Averages**	**Group**	**Factors**	**Averages**	**Group**
T1	10.17(1.37)	a	T1	58.33(31.91)	a
T2	9.92(0.69)	a	T2	58.33(31.91)	a
T3	9.08(1.99)	a	T3	66.67(27.22)	a
T4	10.63(0.64)	a	T4	91.67(16.67)	a
T5	9.92(0.99)	a	T5	75.00(16.67)	a

Values followed by the same letter in the same column do not differ among themselves by the Tukey test in 5% of significance; values in brackets show the standard deviation. (A), Germination speed index; (B), first germination count; (C), average germination time; (D), percentage of germination.

The equation proposed by Maguire (1962) was used in the Germination speed index (GSI). Where G1, G2, ...,Gn are the numbers of calculated seeding from the first, second to the last counting, N1, N2, ..., Nn are the numbers of days of seeding to the first, second to the last counting.

$$GSI = \frac{G1}{N1} + \frac{G2}{N2} + \cdots + \frac{Gn}{Nn}$$

Percentage of germination (G) where N is the number of germinated seed and A is the total number of put seeds (MARA, 1992).

$$G = \frac{N}{A} * 100$$

The average germination time (AGT) was obtained through daily counting of germinated seed to the fifteenth day after the seeding and calculated with the equation proposed by Labouriau (1983), being the expressed results in days. In which ni is the number of germinated seed in the break among each counting and ti is the time that passed in the beginning and i-th counting.

$$AGT = \frac{\Sigma\, ni.ti}{\Sigma\, ni}$$

For the other variables only the 6 more homogeneous seedlings of each treatment were used, these are the variable: radicle length (RL) and aerial part length (CA) - measured with a caliper; leaf fresh mass (LFM); root fresh mass (RFM), leaf dry mass (LDM) and radicle dry mass (RDM) and - the seedling were dried in a greenhouse of forced air circulation on 65°C for 72 h, until it reached a constant weight.

Statistical analysis

The data obtained from the collection of information were analyzed with analysis of variance (ANOVA) to evaluate whether there is any evidence that the sample of the populations of plants differ. This analysis of variance leads to a conclusion that there is evidence that the group of the concentration differ, indicating whether there is a need in investigating which of them are different. This is where the Tukey multiple comparison test is used. The Tukey test compares the difference between each pair of samples with appropriate adjustment for the multiple testing. The test uses tables and comparative letters in columns, meaning equal letters do not differ themselves and columns with different letters have differences in level of 95% confidence.

The results are presented as a matrix showing the result for each pair, either as a P-value ($p < 0.05$), which shows the confidence interval of 95%. The Tukey multiple comparison test and the analysis of variance assumes that the data from the different groups come from populations where the observations have a normal distribution and the standard deviation is the same for each group.

Data were statistically analyzed and were carried out with the Action Stat 3.1 software, using analysis of variance ($p < 0.05$) and Tukey test.

RESULTS AND DISCUSSION

Soy is a worldwide important culture and high economic value; therefore, many researchers study this culture. The allelopathic effect that other plants may cause on soy is relevant because the organic material left by winter crops can harm or benefit the development of the plant. In the familiar agriculture it is even possible to induce aqueous extracts with allelopathic potential on purpose to better develop the plant to avoid the progress of weed (Nóbrega et al., 2009).

As Table 1 shows the germination speed index (A), the first germination count (B), germination average time (C) and percent of germination (D) of soy did not suffer significant changes on influence of aqueous extract of castor beans. Rickli et al. (2011) noticed that the aqueous extract from Neem (*Azadirachta indica*) leaves did not show allelopathic effects when applied to the soy seedling in relation to germination, but the germination

Table 2. Tukey trust break of the parameters evaluated in soy.

LFM (A)			RFM (B)		
Factors	**Averages**	**Group**	**Factors**	**Averages**	**Group**
T1	1.54(0.32)	a	T1	0.61(0.12)	a
T2	1.45(0.30)	a	T2	0.51(0.16)	a
T3	1.06(0.52)	a	T3	0.43(0.20)	a
T4	1.48(0.26)	a	T4	0.77(0.12)	a
T5	1.23(0.15)	a	T5	0.53(0.18)	a
SD (C)			**APL (D)**		
Factors	**Averages**	**Group**	**Factors**	**Averages**	**Group**
T1	1.58(0.07)	a	T1	16.91(2.45)	a
T2	1.77(0.29)	a	T2	16.59(2.16)	a
T3	1.71(0.58)	a	T3	13.93(6.41)	a
T4	2.27(0.46)	a	T4	15.48(1.21)	a
T5	1.88(0.42)	a	T5	14.19(0.45)	a

Values followed by the same letter in the same column do not differ among themselves by the Tukey test in 5% of significance; Values in brackets show the standard deviation. (A), Leaf fresh mass; (B), root fresh mass; (C), stem diameter; (D), aerial part length.

Table 3. Tukey trust breaks of the evaluated parameters in soy.

RL (A)			LA (B)		
Factors	**Averages**	**Group**	**Factors**	**Averages**	**Group**
T1	8.26(1.11)	ab	T1	47.82(9.82)	a
T2	8.72(1.29)	b	T2	44.76(9.20)	a
T3	11.58(4.00)	a	T3	32.80(15.95)	a
T4	9.60(0.32)	ab	T4	45.89(7.99)	a
T5	8.92(0.92)	ab	T5	38.03(4.67)	a
LDM (C)			**RDM (D)**		
Factors	**Averages**	**Group**	**Factors**	**Averages**	**Group**
T1	0.16(0.03)	a	T1	0.05(0.02)	b
T2	0.16(0.02)	a	T2	0.05(0.01)	ab
T3	0.12(0.04)	a	T3	0.05(0.02)	ab
T4	0.16(0.02)	a	T4	0.08(0.02)	a
T5	0.15(0.01)	a	T5	0.06(0.02)	ab

Values followed by the same letter in the same column do not differ among themselves by the Tukey test in 5% of significance; Values in brackets show the standard deviation. (A), Radicular length; (B), leaf area; (C), leaf dry mass; (D), radicle dry mass.

average time and germination average speed were considerable affected by the concentration of 40%, where the average time increased. These authors suggest that these negative influences on the germination average time and germination average speed could harm the agriculture's harvest by changing the uniformity in the production.

The leaf fresh mass (A), root fresh mass (B), stem diameter (C) and Aerial part length (D) of soy did not suffer significant change related to the development when compared to a witness (T1) as shown in Table 2. However, some authors point that the allopathic compounds may show different results not only by concentration, but also by chemical composition (Richardson and Williamson, 1988), and then it was possible to have different results with residues from other castor beans parts and applying aqueous extract of dry leaves.

Results in Table 3 indicate that leaf area and leaf dry mass of soy were not influenced by the introduction of aqueous extract of castor beans leaf. On the other hand, the radicle length and the soy radicle dry mass suffered positive changes when the castor beans aqueous extract was applied. A similar result was found in a work where the radicle increasing growth of soy was observed under the influence of aqueous extract of pine (*Pinus elliottii*) and millet (*Pennisetum glaucum*) (Faria et al., 2009).

Silva et al. (2015) by applying salvia (*Salvia officinalis)*

Table 4. Tukey trust break of evaluated parameters of safflower.

GSI (A)			FGC (B)		
Factors	**Averages**	**Group**	**Factors**	**Averages**	**Group**
T1	3.48(0.48)	a	T1	3.75(0.50)	a
T2	3.84(0.53)	a	T2	3.25(0.50)	a
T3	3.25(2.36)	a	T3	3.50(2.65)	a
T4	1.98(1.22)	a	T4	2.00(1.41)	a
T5	2.88(1.73)	a	T5	3.00(1.41)	a
AGT (C)			**G (D)**		
Factors	**Averages**	**Group**	**Factors**	**Averages**	**Group**
T1	8.04(1.14)	a	T1	57.50(9.57)	a
T2	8.11(0.30)	a	T2	60.00(8.16)	a
T3	7.87(1.50)	a	T3	50.00(33.67)	a
T4	8.74(1.96)	a	T4	37.50(9.57)	a
T5	6.43(1.01)	a	T5	32.50(12.53)	a

Values followed by the same letter in the same column do not differ among themselves by the Tukey test in 5% of significance; Values in brackets show the standard deviation. (A), Germination speed index; (B), first germination count; (C), average germination time; (D), percentage of germination.

leaf extract noticed that there is a stimulation of soy growth, mainly of the radicular part and there was no difference in the germination percentage in relation to the witness with this interference. Nunes et al. (2014) in studies about allelopathic activity of extract from plants covering soy, cucumber (*Cucumis sativus*) and lettuce (*Lactuca sativa*) related that *Crotalaria* is the crop that the extract suffered more positively than other crops, but the linseed extract (*Linum usitatissimum*) showed opposite behavior.

A study done by Peron et al. (2014) obtained that aqueous extract of fresh leaves and dry tobacco (*Nicotiana tabacum* L.) showed a marked inhibitory effect in germination and initial growth of soy seedling. It was also observed that the extracts done with tobacco dry leaves showed more significant effects on germination and speed rate of soy seeds germination, while the extracts made with fresh leaves, as in this work, had more interference in the length root and in the fresh and dry biomass.

With safflower the interferences done with extract concentrations 25, 50, 75 and 100% in relation to germination, considering the averages submitted to analysis of variance and compared by Tukey test with an error rate of 5%. In this study, it was observed that there was no significant difference of development among the seedlings that received the aqueous extract and those which received only water as shown in Table 4.

It can be further noted from Table 4 that trust break to each variable analyzed on germination, germination speed index (A), first germination count (B), average germination time (C) and percentage of germination (D) showed significant changes all the breaks belong to group A.

The aerial part length was the only parameter analyzed that had significant change as item D shows in Table 5. In this case, the treatment T3 the length was the biggest in relation to the other treatments and treatment T2 was significantly smaller than the other treatments.

About the safflower development (*Carthamus tinctorius* L.) in the other parameters of radicular and leaf development there was no positive or negative effect in the treatment with aqueous extract in relation to the witness either, considering the same statistic treatment done with the germination variables. Tables 5 and 6 show the trust break graphics on 95%.

According to Cuchiara et al. (2007) the decomposition of vegetal residuals is one of the most important sources of allelochemicals. In his work, in which he tried to verify the antiproliferative effect of aqueous extract of castor beans (*R. communis* L.) applied to lettuce (*Lactuca sativa* L.), he observed an opposite behavior with extracts done with fresh leaves and extracts done with dry leaves, where the last one showed the biggest allelopathic effect inhibitory in lettuce.

The allelochemial substances in a vegetal depend on the composition, concentration and localization in the plant. These compounds tend to be released in many ways, where ambient variables influence in dispersion (Cuchiara et al., 2007). Correlating with studies already shown, it was analyzed that the extract of castor beans fresh leaf does not have allelopathic effect of safflower plant, but using dry leaved the same result cannot be guaranteed.

Conclusion

The aqueous extract of castor beans positively influenced the development of soy seedlings. The interference

Table 5. Tukey trust break of safflower parameters evaluated.

LFM (A)			RFM (B)		
Factors	**Averages**	**Group**	**Factors**	**Averages**	**Group**
T1	3.76(0.82)	a	T1	0.15(0.03)	a
T2	3.57(1.19)	a	T2	0.14(0.07)	a
T3	4.14(152)	a	T3	0.14(0.05)	a
T4	4.10(1.28)	a	T4	0.16(0.04)	a
T5	3.53(1.49)	a	T5	0.08(0.03)	a
SD (C)			**APL (D)**		
Factors	**Averages**	**Group**	**Factors**	**Averages**	**Group**
T1	4.07(0.77)	a	T1	19.13(2.30)	ab
T2	3.50(0.50)	a	T2	15.61(3.29)	b
T3	3.54(0.44)	a	T3	21.30(2.73)	a
T4	3.24(0.60)	a	T4	19.83(1.75)	ab
T5	3.14(0.32)	a	T5	19.05(3.5)	ab

Values followed by the same letter in the same column do not differ among themselves by the Tukey test in 5% of significance; Values in brackets show the standard deviation. (A), leaf fresh mass; (B), root fresh mass; (C), stem diameter; (D), .aerial part length.

Table 6. Tukey trust break of safflower parameter evaluated.

RL (A)			LA (B)		
Factors	**Averages**	**Group**	**Factors**	**Averages**	**Group**
T1	13.77(3.34)	A	T1	77.26(16.76)	a
T2	15.61(3.29)	A	T2	73.23(24.48)	a
T3	15.98(5.61)	A	T3	84.93(31.29)	a
T4	14.68(4.07)	A	T4	84.19(26.21)	a
T5	12.62(3.70)	A	T5	72.39(30.58)	a
LDM (C)			**RDM (D)**		
Factors	**Averages**	**Group**	**Factors**	**Averages**	**Group**
T1	0.27(0.06)	A	T1	0.02(0.00)	a
T2	0.28(0.10)	A	T2	0.02(0.01)	a
T3	0.29(0.10)	A	T3	0.02(0.01)	a
T4	0.32(0.10)	A	T4	0.02(0.00)	a
T5	0.28(0.11)	A	T5	0.02(0.02)	a

Values followed by the same letter in the same column do not differ among themselves by the Tukey test in 5% of significance; Values in brackets show the standard deviation. (A), Radicular length; (B), leaf area; (C), leaf dry mass; (D), radicle dry mass.

happened mainly in the roots. In concentration T3 the intervention resulted in bigger radicular growth. But in concentration T4 there was the biggest dry mass radicular. Considering the obtained results by statistic treatments, it is concluded that extract of castor beans fresh leaf (*R. communis* L.) applied to the beginning stage of safflower (*C. tinctorius* L.) showed stimulant effects of leaf area with extract T3 (50%). It is suggested for further studies the elaboration of aqueous extract with castor beans dry leaves to verify the allopathic effect of it in soy and safflower crop.

Conflicts of Interests

The authors have not declared any conflict of interests.

REFERENCES

Ahmed MZ, Omran MF, Mansour SZ, Ibrahim NK (2007). Effectiveness of *Carthamus tinctorius* L. in the restitution of lipid composition in irradited rats. J. Radiat. Res. Appl. Sci. 20(1):75-94.

Babula P, Adam V, Kizek R, Sladký Z, Havel L (2009). Naphthoquinones as allelochemical triggers of programmed cell death. Environ. Exp. Bot. 65(2-3):330-337.

CONAB (Companhia Nacional de Abastecimento) (2016). Acompanhamento da safra brasileira: grãos. Boletim informativo. Décimo segundo levantamento. Safra 2015/2016. Brasília DF3(12):184.

Costa AG, Sofiatti V, Maciel CD, Poletine JP, Sousa JI (2014). Weed management strategies for castor bean crops. Acta Sci. Agron. 36(2):135-145.

Cuchiara CC, Borges CS, Sopezki MS, Souza SAM, Bobrowski VL (2007). Efeito antiproliferativo dos extratos aquosos de mamona (*Ricinus communis* L.). Rev. Bras. Biocienc. 5(2):639-641.

Davood MY, Karimmojeni H, Khodaee MM, Sabzalian MR (2013). A

bioassay assessment of safflower allelopathy using equal compartment agar methods. J. Agrobiol. 30(2):97-106.
FAO (Food and Agriculture Organization) (2016). OECD-FAO Agricultural Outlook 2016-2025. OECD-FAO Agricultural Outlook.1(18):1-137.
FAOSTAT (Food And Agriculture Organization Of The United Nations) (2013). Crops 2013. Available at: http://faostat.fao.org/. Access: 05. Sep. 2016.
Faria TM, Gomes Júnior FG, Sá ME, Cassiolato AMR (2009). Efeitos alelopáticos de extratos vegetais na germinação, colonização micorrízica e crescimento inicial de milho, soja e feijão. R. Bras. Ciênc. Solo 33(6):1625-1633.
Furtuoso COM, Guilhoto JJ (2013). Estimativa e mensuração do produto interno bruto do Agronegócio da economia brasileira, 1994 a 2000. Rev. Econ. Sociol. Rural 33:4.
Guidorizzi FVC (2016). Acúmulo de macronutrientes e produtividade de genótipos de cártamo (*Carthamus tinctorius* l.) em função da adubação nitrogenada no sistema plantio direto.Botucatu, Universidade Estadual Paulista. Dissert. Mestrado Agron.69 p.
Kpanja EJ, Omage JJ, Bawa GS (2016). Performance, bio-chemical and haematological characteristics of broilers fed graded levels of 30 minutes steam-boiled castor bean cake based diets. J. Anim. Prod. Res. 28(1):130-137.
Labouriau LG (1983). A germinação das sementes.Washington: Secretaria Geral da Organização dos Estados Americanos. 174p.
Maguire JD (1962). Speed of germination-aid in selection and evaluation for seedling emergence and vigor. Crop Sci. 2(1):176-177.
MARA (Ministério da Agricultura e Reforma Agrária) (1992). Regras para análise de sementes. Brasília: SNDA / DNDV / CLAV. 365 p.
MARA (Ministério da Agricultura e Reforma Agrária) (2009). Regras para Análise de Sementes. 499 p.
MARA (Ministério da Agricultura e Reforma Agrária) (2013). Balança comercial total e agrícola. 16p.
Nóbrega LHP, Lima GP, Martins GI, Meneguetti AM (2009). Germinação de sementes e crescimento de plântulas de soja (*Glycine max* L. Merrill) sob cobertura vegetal. Acta Sci. Agron. 31(3):461-465.
Nunes JVD, Melo DD, Nóbrega LHP, Loures NTP, Sosa DEF (2014). Atividade alelopática de extratos de plantas de cobertura sobre soja, pepino e alface. Rev. Caatinga 1(27):122-130.
Peron F, Rodrigues MS, Souza GAB, Lúcio LC, Bido GS (2014). Efeitos alelopáticos de extratos de tabaco sobre o desenvolvimento inicial de soja. SaBios: Rev. Saúde Biol. 9(1):53-60.
Richardson DR, Williamson GB (1988). Allelopathic effects of shrubs of the sand pine scrub on pines and grasses of the sand hills. For. Sci. 34(1):592-596.
Rickli HC, Fortes AMT, Silva PSS, Pilatti DM, Hutt DR (2011). Efeito alelopático de extrato aquoso de folhas de Azadirachta indica A. Juss. em alface, soja, milho, feijão e picão-preto. Semina: Ciênc. Agrár. 32(2):461-472.
Rizvi SJH, Haque H, Singh UK, Rizvi V (1992). A discipline called allelopathy. Allelopathy: Basic and applied aspects. Chapman & Hall, London, England.
Santos VM, Castro HG, Cardoso DP, Lima SO, Leal TCAB, Santos GR (2011). Avaliação do crescimento e da produtividade da mamoneira BRS 149 Nordestina em dois níveis tecnológicos. J. Biotechnol. Biodivers. 2(3):58-66.
Sehgal D, Raina S N (2005). Genotyping safflower (*Carthamus tinctorius*) cultivars by DNA fingerprints. Euphytica 146(1-2):67-76.
Silva ALK, Silva KG, Paulert R, Zonetti PC, Albrecht LP (2015). Germinação e crescimento inicial de plântulas de euphorbia heterophylla L. e *Glycine max* L. Merril na presença de extratos foliares de *Salvia officinalis* L. Revista em Agronegocio e Meio Ambiente 8(2):291-301.
Silva C J (2013). Caracterização agronômica e divergência genética de acessos de cártamo. Tese (Doutorado em Agronomia - Agricultura) - Faculdade de Ciências Agronômicas, Universidade estadual Paulista, Botucatu. pp. 1-51.

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