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Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Molecular cloning and expression of a novel gene related to legume lectin from *Salvia miltiorrhiza* Bunge

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Lectins have been well studied and proved to play important roles in plant defense but information of legume lectins from non-legume plants has been rarely reported. A new legume lectin gene, designated as *SmL*1, was cloned from *Salvia miltiorrhiza* Bunge, a famous traditional Chinese medicinal plant. The cDNA of *SmL*1 was 919 bp in length and contained an 822 bp open reading frame (ORF) encoding a putative lectin precursor with two legume lectin domains. The deduced SML1 protein of *SmL*1 shared 29 to 43% identities with other legume lectin sequences. Real time PCR analysis revealed that *SmL*1 was predominantly expressed in the leaves and could be induced by pathogens and MeJA. The recombinant protein (rSmL1) of *SmL*1 in *Escherichia coli* M15 was purified and showed agglutination activity towards rabbit and mouse red blood cells, and anti-bacterial activity against *E. coli* (ATCC35218), *Pseudomonas lachrymans* (PSL) and *Xanthomonas campestris* pv. Campestris (Pammel) Dowson (XC-1). Based on these results, SmL1 could play a role in medicinal plant disease control.

Key words: Anti-bacterial activity, gene expression, legume lectin, recombinant protein, *Salvia miltiorrhiza* Bunge.

INTRODUCTION

Plant lectins or agglutinins are a large group of proteins, which possess at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharide (Carlini and Grossi-de-Sa, 2002; Peumans and Van Damme, 1995). Lectins exist in most living organisms but were first identified as plant proteins that agglutinate human red blood cells (Van Damme et al., 1998). Now thousands of plant lectins were found and stored in the Lectin database (Lectindb, http://proline.physics.iisc.ernet.in/cgi-bin/lectindb/). Based on their different carbohydrate-binding specificities, plant lectins have been divided into 12 different families, such as (1) *Agaricus bisporus* agglutinin homologs, (2) Amaranthins, (3) Class V chitinase homologs with lectin activity, (4) Cyanovirin family, (5) EEA family, (6) GNA family, (7) proteins with hevein domains, (8) Jacalins, (9) proteins with legume

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Abbreviations: EAPL, Extralong autumn purple bean; **WGA,** wheat germ agglutinin; **ORF,** open reading frame.

lectin domains, (10) LysM domain, (11) Nictaba family (formerly Cucurbitaceae phloem lectins), and (12) Ricin-B family (Van Damme et al., 2008). It has been suggested that plant lectins play important roles due to their abundance in the immune defence, and also that lectins have been coopted to adapt for several functions during evolution (Charungchitrak et al., 2011). More and more attention has been draw onto the antimicrobial activity of plant lectins. Many lectins had been found with anti-microbial activity, such as lectins derived from *Phthirusa pyrifolia* leaves (Costa et al., 2010), *Eugenia uniflora* (EuniSL) (Oliveira et al., 2008) and *Myracrodruon urundeuva* (Sá et al., 2009). In recent years, even some lectins have anti-viral activity, such as lectin from seeds of *Phaseolus vulgaris* L. cv. Extralong Autumn Purple Bean (EAPL) and BanLec isolated from the fruit of bananas, indicated Anti-HIV-1 activities (Fang et al., 2010; Swanson et al., 2010). Some of them, such as a wheat germ agglutinin (WGA) (Ciopraga et al., 1999), Concanavalin A (Safina et al., 2008), *Sebastiania jacobinensis* bark lectin (SejaBL) (Vaz et al., 2010) and *P. pyrifolia* leaf lectin (Costa et al., 2010), have been tried to biologically control plant diseases.

However, different families of plant species, as well as different tissues within the same plant, can contain different lectins with different bioactivities, including different carbohydrate-binding specificities and antimicrobial activity (Charungchitrak et al., 2011). They occur widely in plants but manifest significant differences in bioactivities, which means only a few of them has application prospect. Presently, most of their functions are still unclear in many plants. *Salvia miltiorrhiza* Bunge is a well-known medicinal plant in the Labiatae family. Its dry roots or rhizomes (called 'danshen' or 'tanshen' in China, but better known in the west as Chinese sage or red sage root) has been used in the treatment of cardiovascular, cerebrovascular, hyperlipidemia, and acute ischemic stroke diseases for decades (Kang et al., 2003; Yang et al., 2006; Zhong et al., 2009). One soil-borne disease induced by *Fusarium solani* could lead to rotten roots of *S. miltiorrhiza*, which results in the reduction of yields and the decline of quality. In recent years, the incidence of diseased plants in the field has varied from 10 to 30% in its growth zones. It is difficult to control this disease by farm chemicals, which may pollute this medicinal crop and its environment if applied irrationally. Biocontrol means has attracted people's attention in sustainable environmental development to control this disease compared to the application of chemicals.

To explore the potential lectin protein-encoding genes, we screened our cDNA library of *S. miltiorrhiza* (Hua et al., 2011) and found 30 unigenes encoding putative lectin proteins. Contig1927 (one of lectin unigenes) has also been reported as the highest abundance gene in *S. miltiorrhiza* root EST library (Li et al., 2010). Taking into account that the roots are the part of occurrence of rotten root in *S. miltiorrhiza*, we chose this gene as our object in the present paper. Firstly, we cloned and characterized this novel gene (GenBank Accession Number: EF593952), then we over-expressed it in *Escherichia coli*, eventually its agglutination and antibacterial activity had been identified *in vitro*. The purified recombinant protein showed significant anti-bacterial activity against *E. coli* (ATCC35218), *Pseudomonas lachrymans* and *Xanthomonas campestris pv*. Campestris (Pammel) Dowson. Therefore, these results suggested that the application of this gene in genetically modified plants may be an efficient way to control root rot in *S. miltiorrhiza* and generate more profitable and productive yields without affecting environment.

MATERIALS AND METHODS

Plant materials

The seeds of *S. miltiorrhiza* Bunge were collected from Shangluo County, Shaanxi Province, China. The plants were grown in pots with soil in greenhouse under normal irrigation and fertilization. Two-month-old seedlings were used for DNA extraction and the following two treatments, respectively. For the methyl jasmonate (MeJA) treatment, leaves were sprayed with 5 μM MeJA. Then samples were collected at hour 0, 1, 6, 12, 24 and 48 h after MeJA application. Young leaves nicked with a knife were infected by *P. lachrymans* (PSL, 10^8 cfu·ml^{-1}), and collected at 0, 24, 48, 72 and 96 h post-infection. All collected samples were frozen immediately in liquid nitrogen and stored at -80°C before use.

Isolation of the lectin gene

The genomic DNA was extracted from *S. miltiorrhiza* by CTAB based method (Beji et al., 1987). Total RNAs were extracted respectively from the roots, stems and leaves of control and treated plantlets, using BIOZOL Reagent (BIOER, China) according to the manufacturer's instructions. The first strand cDNA was synthesized using Revert Aid First Strand cDNA Synthesis Kit (MBI, Fermentas). The lectin gene was amplified with the forward primer: 5′-ATGGCCAAGCTTCTCCAAAAC-3′ and the reverse primer: 5′-GTCGATCGCTTAGTCCTTATTGA-3′, both of which were designed according to unigene sequence (Contig1927) from cDNA library of *S. miltiorrhiza*. The PCR reaction was performed under the following conditions: genomic DNA or cDNA was denatured at 94°C for 4 min followed by 30 cycles of amplification (94°C for 30 s, 54°C for 30 s and 72°C for 80 s) and then extention at 72°C for 10 min. The PCR fragments were purified by DNA Gel Extraction Kit (Biospin) and inserted into pGMT-Easy T vector (Promega, USA) and sequenced.

Expression level of *SmL1* gene in different tissues

SYBR Green II dye (Takara, Japan) was used for detecting the expression levels of *SmL*1 under various treatments or in different tissues of *S. miltiorrhiza*. Real-time PCR was conducted with an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad). The *SmL*1 was amplified with the forward primer: 5′-CATGACATC GTCTCGTGGTATTTC-3′ and the reverse primer: 5′-GATCGCTTAGTCCTTAT TGATTTGC-3′. A housekeeping gene, *SmGAPDH* (glyceraldehydes-3-phosphate dehydrogenase, CV170251), was used as our control and was amplified with forward primer GAPF 5′-CCACCGTCCACTCCATCACT-3′ and

reverse primer GAPR (5′-TG GGAACTCGGAACGACATAC-3′). The amplification of *SmL*1 and *SmGAPDH* gene was performed as: cDNA was denatured at 95°C for 5 min followed by 40 cycles of amplification (95°C for 10 s, 59°C for 15 s and 70°C for 10 s to collected fluorescence). The products were validated by electrophoresis on a 1.5% agarose gel then further sequenced for confirmation. Expression was quantified by the comparative C_T method (Vandesompele et al., 2002). Each data point represented the average of three separate experiments. Statistical analysis was done with SPSS 13.0 software. One-way analysis of variance (ANOVA) and Tukey's pair-wise comparison tests (P<0.05) were successively performed to determine significant differences between means.

Construction of lectin expression vector

The mature protein-encoding region was amplified using the forward primer: 5′-GGGGTACCCAAACGACGTCCTTCACCTA-3′ containing a *Kpn*I restriction site (underlined) and the reverse primer 5′-AACTGCAGGTCGATCGCTTAGTCC TTATTGA-3′ containing a *Pst*I restriction site (underlined). The amplification condition was as described previously. The PCR product digested with *Kpn*I and *Pst*I was ligated with pQE30 vector using T4 DNA ligase (TaKaRa), and then transformed into *E. coli* M15 strain. Positive transformants were screened by plating on Luria-Bertani (LB) agar containing kanamycin and ampicillin antibiotics after growing overnight at 37°C.

SmL1 gene expression and purification

The positive M15 cells were cultured in LB medium containing 100 mg/ml ampicillin and 50 mg/ml kanamycin at 37°C to an absorbance of 0.6 to 0.8 at 600 nm. 1 mM IPTG were applied into culture to induced target gene expression. The cells for SDS-PAGE were harvested at 0, 1, 2, 3, 4, 5 and 6 h after induction, then centrifuged at 4°C, 4,000 g for 8 min. Afterwards, the pellet with 6 h induction was re-suspended in PBS buffer (containing 8 M urea), then the cell walls were fractured by ultrasound (400 w for 7 min). After another centrifuge at 4°C, 10,000 *g* for 20 min, the supernatant was collected for protein purification using equilibrated His-bond Ni Affinity Resin column (Zhuoguan, China) according to the manufacturer's instructions. The elutions were analyzed by SDS-PAGE.

Agglutination activity test of recombinant protein of *SmL1* gene

The erythrocytes from rabbit and mouse (pre-treated with trypsin) were washed with normal saline (0.9%) for three times. The recombinant protein (0.5 mg/ml) solution was serially diluted with two-fold increments. Agglutination assays were carried out in a 96-U-well plate in a final volume of 50 µl containing 25 µl the diluted recombinant protein solutions and 25 µl of a 1% suspension of red blood cells. Elution/washing buffer of the recombinant protein and normal saline were considered negative controls. Agglutination was assessed visually after 2 h at room temperature using microscope. Two separate experiments for every individual were performed and the means were calculated.

Anti-bacterial activity of recombinant protein of *SmL*1 (rSmL1) gene

The anti-bacterial activity of rSmL1 was qualitatively evaluated by optical density. *E. coli* (ATCC35218), PSL, and XC-1 were bought from China General Microbiological Culture Collection Center as test bacteria. The test bacteria were incubated at 28°C for PSL and XC-1, at 37°C for *E. coli* (ATCC35218) overnight at 180 rpm. A secondary propagation of the cells was carried out for another 2 to 3 h, then cultures were divided into equal parts (50 ml for each). Equal amount of purified rSmL1 protein was added into each culture, and boiled purified protein was used as negative control. After inoculation of purified recombinant rSmL1, the density of each medium was measured at OD 595 nm after 0, 1, 2, 3, 4 and 5 h. The experiments were done three times. The *E. coli*, PSL, and XC-1 were cultured in Ordinary Broth Medium at 37°C, KB medium without agar at 28°C, and modified Broth Medium containing sucrose (10 g/L) at 28°C, respectively. Significant differences between treatment and control groups were analyzed using one-way ANOVA with SPSS13.0 software.

RESULTS AND DISCUSSION

Isolation and sequence analysis of *SmL1*

The full-length cDNA of *SmL*1 was amplified by RT-PCR. The cDNA fragment was 919 bp in length and contained an 822 bp open reading frame (ORF) positioned from 33 to 855 bp (Figure 1a). The ORF of *SmL*1 encodes 273 amino acids with the isoelectric point of 5.00 and molecular mass of 29.2 kDa. No intron was found in *SmL*1 sequence by comparing genomic and cDNA sequences. The deduced amino acids sequence (named as SmL1) had two legume lectin domains, a lectin-legB (amino acids 28 to 203) domain, and a lectin-legA (amino acids 211 to 256) domain according to BLASTp search against NCBI (Figure 1b). The amino acid number and molecular mass were identical with other legume lectins as previously reported (Etzler, 1985; Sharon and Lis, 1990; Van Damme et al., 1998). Protein-protein BLAST of deduced *SmL*1 amino acid sequence showed 29 to 43% identities and 49 to 61% positives in local alignments against candidate genes from *Arachis hypogaea* (ABJ15831), *Sophora flavescens* (AF285121), *Sophora alopecuroides* (AAA74572), *Glechoma hederacea* (AAN 050977), *Canavalia ensiformis* (CAA25787), *Cladrastis kentukea* (Q39529), *Sophora japonica* (AAB51442) and *Phaseolus leptostachyus* (CAH60215). All these results suggested that SmL1 belonged to legume lectin family.

Most legume lectins are extensively distributed in legume plants. However, Gleheda (AAN05097) (a legume lectin isolated from *G. hederacea*) and SBoL (a *Salvia bogotensis* seed lectin) had been identified from non-legumes (Vega and Pérez, 2006; Wang et al., 2003) and some of which has been shown to have important physiological activity in plants, such as Gleheda, which indicate insecticidal activity against Colorado potato beetle larvae (Wang et al., 2003). To our knowledge, this is the first time to report legume lectin gene in *S. miltiorrhiza.* And our lectin gene encoding protein sequence was similar to Gleheda, which indicated that it may also played unusual roles in physiology in

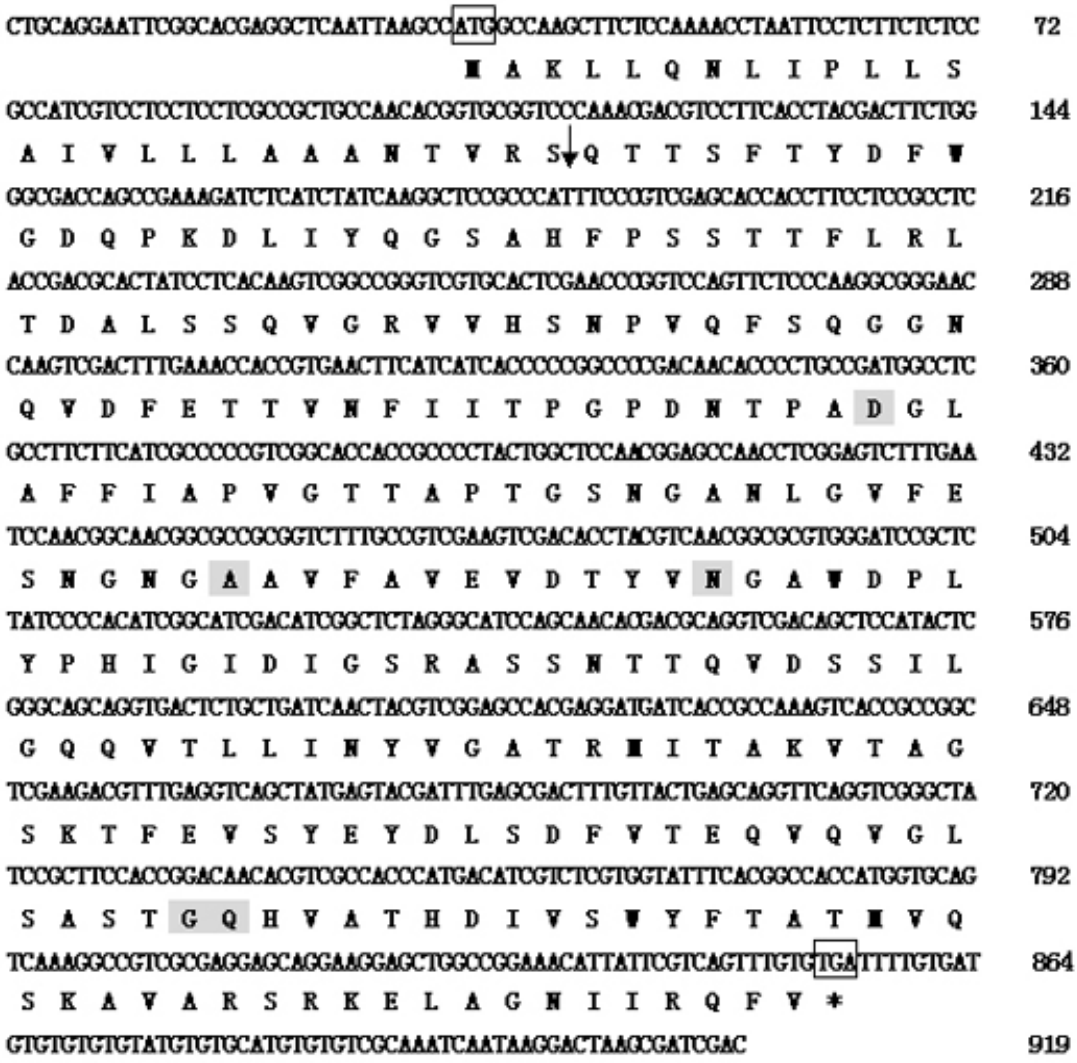

Figure 1a. The full-length cDNA sequence and deduced amino acid sequence of *S. miltiorrhiza* lectin (*SmL*1). The star codon (ATG) and the stop codon are shown by boxes. The putative processing sites for the cleavage of in C-terminal are marked by arrow. The putative N-glycosylation site is shown by black ground.

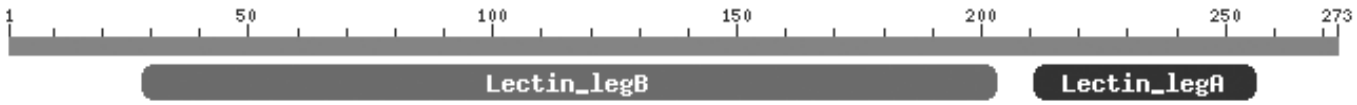

Figure 1b. Putative conserved domain has been detected with the protein-protein BLAST tool of NCBI.

S. miltiorrhiza as Gleheda in *G. hederacea.*

The signal peptide (SignalP, http://www.cbs.dtu.dk/services/SignalP/) prediction on SmL1 revealed a single peptide cleavage site between position 27 and 28th amino acid residues (Ser-Gln) (Figure 1a). The cleavage of the signal peptide in C-terminal sequence of SmL1 resulted in a lectin polypeptide of approximately 26.3 kDa with theoretical pI 4.76.

Sequence analysis of SmL1

Multi-alignment of SmL1 with other legume lectins (Figure 1) was conducted by ClustalW (Figure 2a). SmL1 showed 43, 37, 37 and 37% identity to protein sequences of Gleheda*, S. alopecuroides* lectin, *S. flavescens* lectin, and LecClAII (*C. kentukea* agglutinin), respectively. A further examination on the sequences of SmL1, Gleheda,

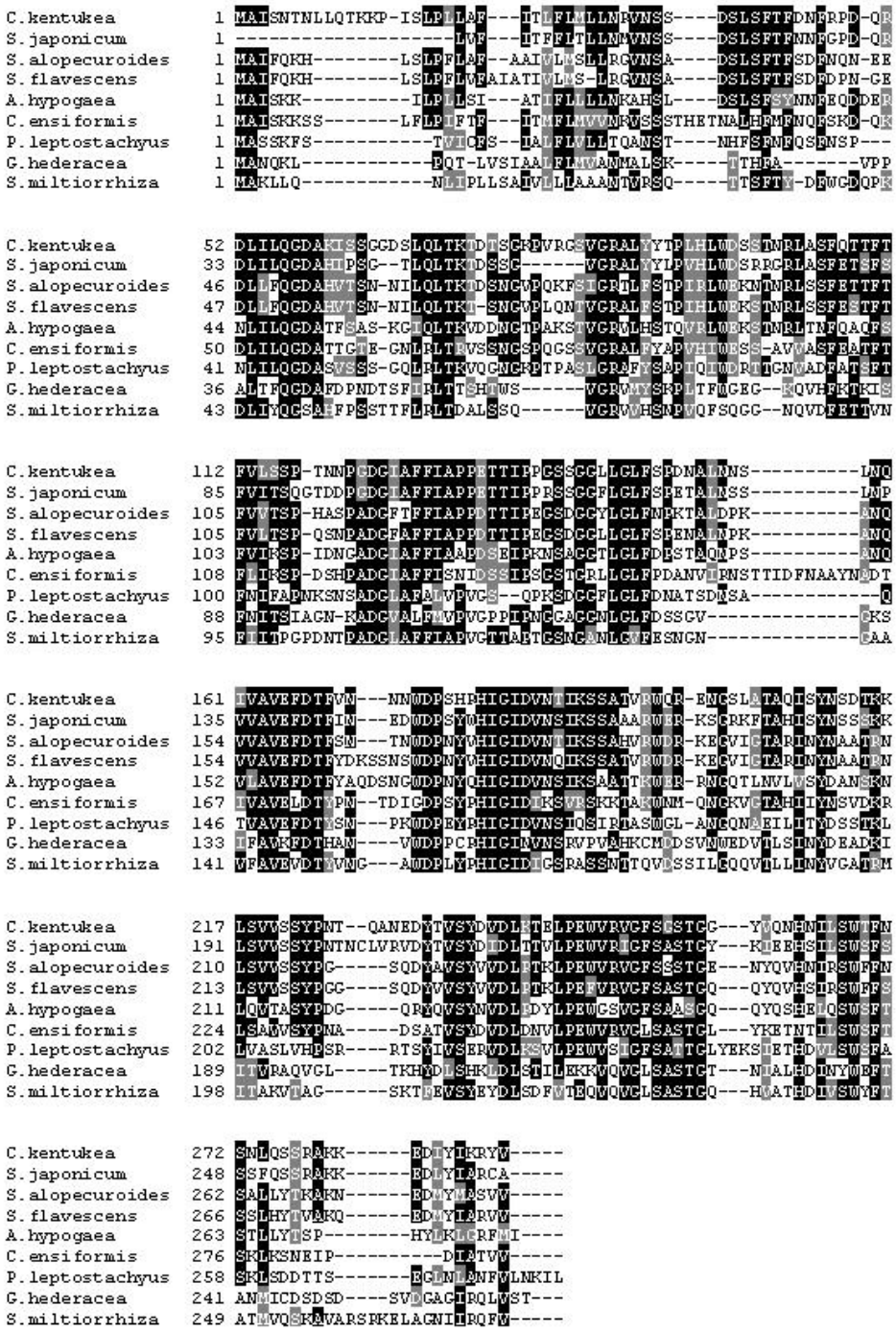

Figure 2a. Multiple sequence alignment of SmL1 with other legume lectin. The multiple sequence alignment was performed by Clustal W (http://www.ebi.ac.uk/clustalw/index.html): *C. kentukea* Agglutinin (Q39529); *S. japonicum* (AAB51442); *S. alopecuroides* lectin (AAA74572); *S. flavescens* (AF285121); *A. hypogaea* (ABJ 15831); *C. ensiformis* (CAA25787); *P. leptostachyus* (CAH60215); *G. hederacea* lectin (AAN050 977); *S. miltiorrhiza* (EF593952).

S. flavescens lectin, and *A. hypogaea* lectin, indicated that the residues forming the mono-saccharide binding sites were highly conserved. The putative carbohydrates binding sites in SmL1 were found as: Asp_{107}, Ala_{139}, Asn_{151}, Gly_{234}, Gln_{235} and His_{236} (Figure 1a). Asp_{107}, Asn_{151}, Gly_{234} were identical to Gleheda, Gln_{235} was

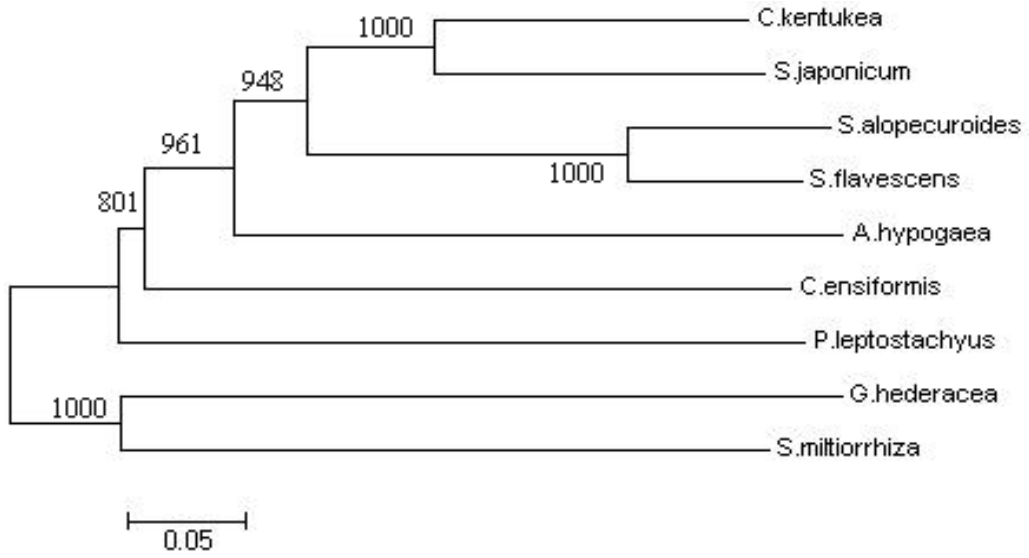

Figure 2b. The phylogenetic relationships of SmL1 with other related proteins. The tree was constructed the alignment resulting from analysis by MEGA4.0.

identical with *S. flavescens* lectin and *A. hypogaea* lectin (Liu et al., 2008). Ala_{139} in SmL1 was replaced by Gly_{93} compared to Gleheda (Wang et al., 2003). Some amino acid residues of monomer ConA (a classical legume lectin from *C. ensiformis*) and legume lectin LoLI from *L. odoratus* L. were highly conserved, which decided the sorts of binding carbohydrate, such as Asp_{81}, Gly_{99}, Asn_{125}, Gly_{208}, Ala_{20s9} and Glu_{210} in LoLI protein. Asp_{81} and Asn_{125} in LoLI were consistent in all known legume lectins, and necessary for carbohydrate activity; while other amino acid residues, Ala_{209} and Glu_{210} (LoLI), were alterable, probably participated in binding carbohydrate (Bourne et al., 1990; Perçin et al., 2012). Some amino acid residues (Asp_{107}, Asn_{151}) of the putative conservative carbohydrates binding sites in SmL1 were invariant, while Ala_{139} and His_{236} were different from other legume lectins, just like ConA and LoLI (Sharon and Lis, 1990).

Furthermore, the phylogenetic relationship of SmL1 with other candidates was determined. The phylogenetic tree was constructed by neighbor-joining method with MEGA 4.0. It shows the SmL1 and Gleheda were clustered into one sub-group; other lectins from legume plants were clustered into another sub-group but SmL1 was closer to legume lectins than Gleheda in molecular evolution (Figure 2b). The amino acid residues on carbohydrate-binding site (in comparison with Gly_{93} in Gleheda, Ala_{139} in SmL1 were more conserved among legume lectins) also supported our assumption. The identification of *SmL*1 gene from *S. miltiorrhiza* showed high similarity with Gleheda, provided another direct evidence that the possibility of finding an ortholog of legume lectins gene outside the family Fabaceae (Wang et al., 2003), which also indicated the evolutionary processes of the same ancestor of modern legume lectins.

Tertiary structure prediction of *SmL*1 amino acid sequence

According to the deduced amino acid sequence of *SmL*1 gene, the three-dimensional model of SmL1 was constructed using SWISS-MODEL (http: //swissmodel.expasy.org/) (Figure 3). The model indicated that the SmL1 monomer consisted of two β-sheets, a curved seven-stranded β-sheet forming the front face, and a flattened six-stranded β-sheet forming the back face of the monomer, which interconnected by turns and loops. Additionally, a four-stranded β-sheet, referred as the S-sheet, was occurred between the two β-sheets at the top of the monomer. The model also showed SmL1 can bind calcium and manganese ions, which could keep the amino acid residues of the sugar-binding site at the required positions (Roopashree et al., 2006). Like other legume lectins (Loris et al., 1998; Sharon and Lis, 1990; Varrot et al., 2011), β-sheets in SmL1 are dominated structure, whereas α-helices are virtually absent. Therefore, it can be concluded that SmL1 adopts the same β-sandwich structure as the classical legume lectins (Wang et al., 2003). Most known legume lectins are homodimers or homotetramers (Li et al., 2012). One monomer cannot form the complex structure with carbohydrates (Eijsden et al., 1992; Li et al., 2012), whether the SmL1 is homodimers or homotetramers still need to be further elucidated.

Expression patterns of *SmL*1 in *S. miltiorrhiza*

According to the report of Li et al. (2010) *SmL*1 have a very high expression level in the *S. miltiorrhiza* root (2010). However, our results of real-time PCR shows that

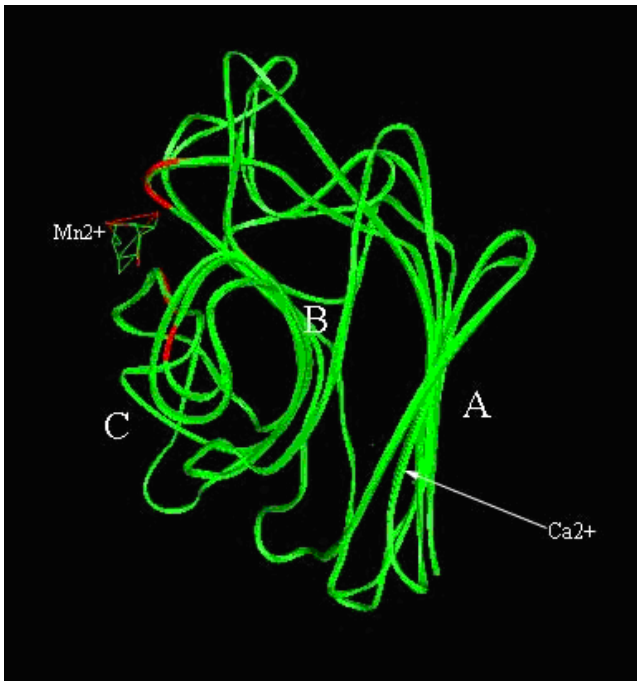

Figure 3. Tertiary structure prediction of *SmL*1 amino acid sequence. **A.** flattened six-stranded β-sheet. **B.** Seven-stranded β-sheet, **C.** The S-sheet. The red fragments on the backbone show the putative carbohydrates binding sites.

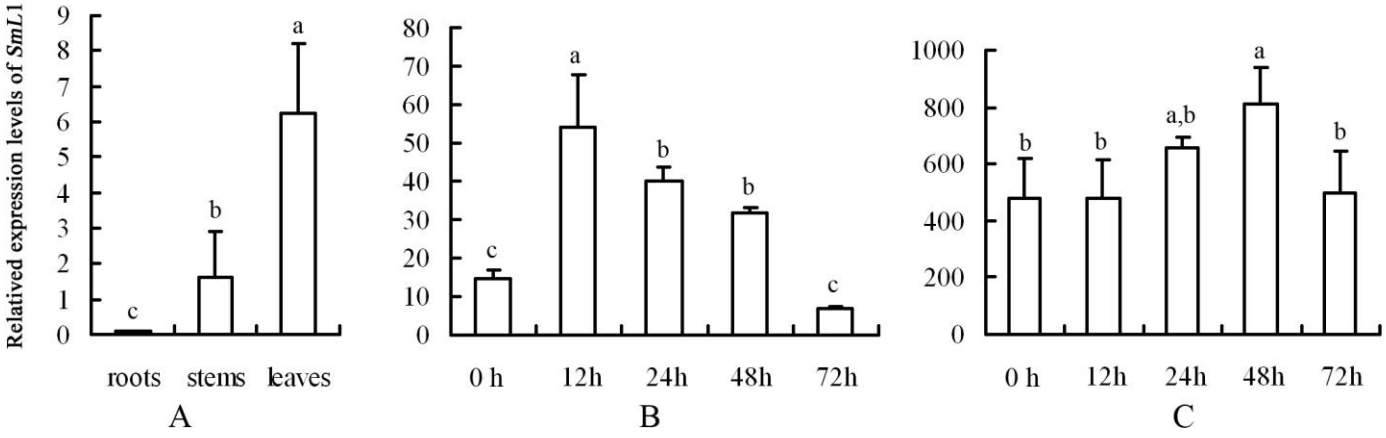

Figure 4. Result of the real-time PCR shows *SmL*1 gene expression in different tissues **(A)** or under different treatments **(B)** PSL, **(C)** MeJA). Bars with the same lowercase letter are not significantly different (P > 0.05).

the expression level of SmL1 was expressed highly in leaves, and but low in stems and roots. The expression level of SmL1 in leaves was approximately 4 times as high as that in stems, and little mRNAs was of *SmL1* were detected in roots (Figure 4A). *Gleheda gene* is also predominantly expressed in the leaves, which encoding the closer legume lectin protein of SmL1 (Wang et al., 2003). This might implied that *SmL1* has high expression levels in various organs, and plant lectins may have important roles according to their abundance. Many legume lectins were served as defense molecules against insect herbivores and pathogens. And the lectin protein-encoding genes can also be induced by insect attack or pathogen infection. To investigate whether *SmL*1 expression can be induced by pathogens, we firstly selected XC-1(a pathogen causing black rot of cabbage)

to infect two-month-old *S. miltiorrhiza* seedlings. After XC-1 infection, *SmL*1 expression was induced to the highest level at 12 h, and gradually returned to normal levels within 72 h (Figure 4B). That result indicated that *SmL*1 was involved in defense against pathogens in *S. miltiorrhiza*.

Jasmonates, as important signal molecules of plant responses to abiotic and biotic stresses, regulate induced defense mechanisms in plants after insect attack and wound response in general (Wasternack et al., 2006). Moreover, the previous studies had showed that the increase of jasmonate levels and the expression of wound-inducible genes after herbivory is a common phenomenon in many plant systems (Qu et al., 2004; Schmidt et al., 2004; Vandenborre et al., 2009). So, we also further determined the change of *SmL1* transcripts level after exposure to methyl jasmonate (MeJA) a derivative of jasmonates) in *S. miltiorrhiza*. Treatment with MeJA only slightly increased transcripts transcript abundance with expression peaking after at 48 h after treatment, and the maximum peak were only about 1.6-fold higher when compared with the control (Figure 4C). So, we concluded that *SmL1* is mainly involved in response against pathogen and may play a small limited role in defense against insect herbivores.

Agglutination and antimicrobial activity test

The result of SDS-PAGE indicated that *SmL*1 gene expressed a protein (named as rSmL1) with the molecular weight of about 26.2 kDa (Figure 5), which was identical with the size of SmL1 mature monomer predicted by bioinformatics method. The agglutination activity assays showed that the recombinant rSmL1 protein could agglutinate mouse and rabbit red blood cells compared to negative control. The minimal concentration required to agglutinate trypsin-treated mouse and rabbit erythrocytes was about 1.99 and 3.91 µg/ml, respectively. Obviously, the agglutination potential of rSmL1 was weaker than ConA (0.98 µg/ml) and Gleheda (0.22 µg/ml) (Jiang et al., 2006; Wang et al., 2003). This may be caused by applying more tyrpsin in our experiment. The bacterial growth experiments showed that the purified protein could inhibit the growth of *E. coli* (ATCC35218), PLS and XC-1 (Figure 6). When the concentration of recombinated SmL1 increased to 4 µg/ml, *E. coli* barely showed any growth (Figure 6A and B). Compared to *E. coli*, the inhibition effects to PLS (Figure 6C) and XC-1 (Figure 6D) could be observed when the medium contained 5 µg/ml purified proteins. This also further elucidated its role in the anti-pathogen responses in *S. miltiorrhiza*.

Conclusion

Plant lectins have been studied over a century. Legume lectin family is the best known lectin family from plants,

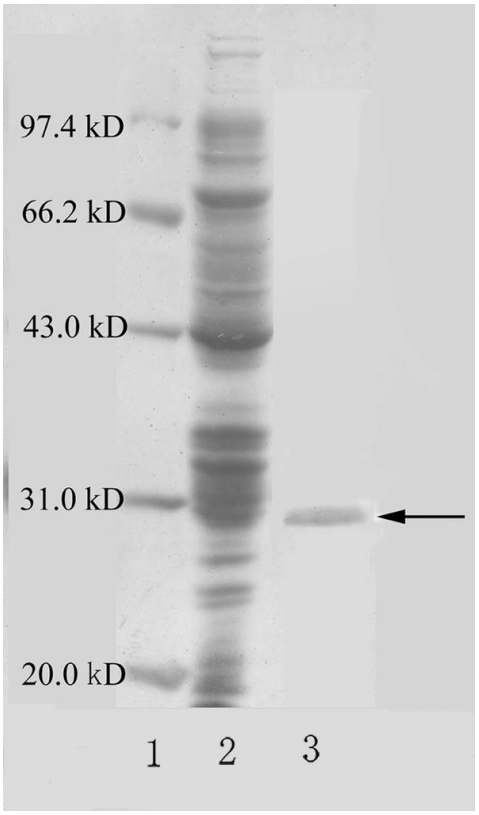

Figure 5. SDS-PAGE analysis of recombinant rSmL1 protein. Line 1, protein marker; line 2, the protein induced after 5 h; line 3: the purified protein (rSmL1).

which are usually, but not exclusively, found in the leguminous plants. In recent years, legume lectins also occur in several non-leguminous species, such as *G. hederacea* and *S. bogotensis*. But limited numbers of legume lectins from non-legume plant were reported. Now, we cloned and characterized a legume lectin gene, *SmL1*, which is expresses abundant in *S. miltiorrhiza* root. And *SmL1* can be induced by pathogen. Its purified recombinant protein from *E. coli* showed significant agglutination and antibacterial activity *in vitro*. These facts indicated that the SmL1 protein might be involved in the defense of plant against pathogen. The application of this gene in future plant genetic modification may be an efficient way to control root rot without damaging the environmental biodiversity.

Conflict of interests

The author(s) did not declare any conflict of interest.

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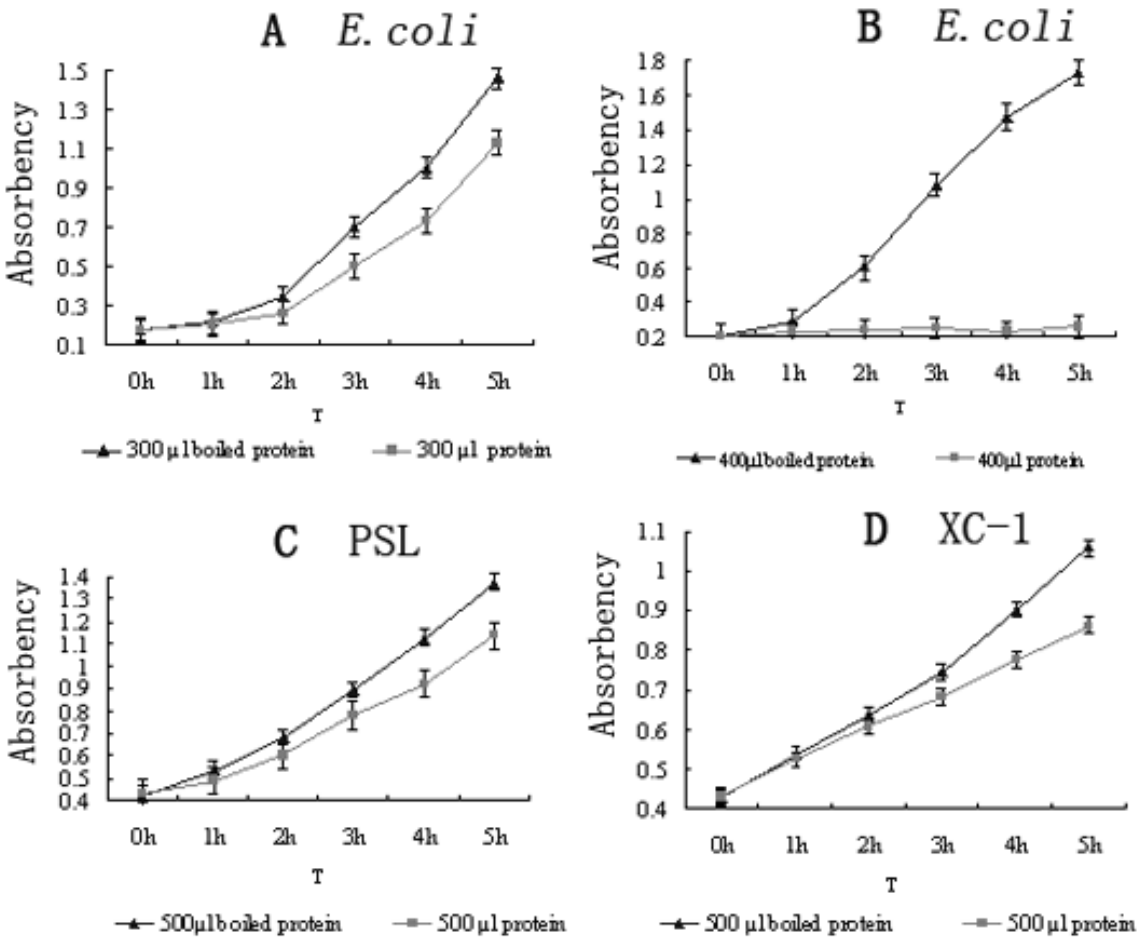

Figure 6. The purified protein inhibits the growth of bacterium. **A)** The growth states of *E. coli* with 2 μg/ml rSmL1 protein. **B)** The growth states of *E. coli* with 4 μg /ml rSmL1 protein. **C)** The growth states of PSL with 4 μg/ml rSmL1 protein, **D)** is the growth states of XC-1 with 4 μg /ml rSmL1 protein.

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Full Length Research Paper

Molecular screening for erythromycin resistance genes in *Streptococcus pyogenes* isolated from Iraqi patients with tonsilo-pharyngites

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Streptococcus pyogenes **is the leading cause of uncomplicated bacterial pharyngitis and tonsillitis commonly referred to as strep throat. Erythromycin is administered for patients allergy to penicillin. In this study, 125 throat swab samples were collected from children between 2-12 years old with tonsillo-pharyngitis attended to at the AL-Imammain AL-Kadhimain Medical City-Baghdad-Iraq and Pediatric Caring Hospital-Baghdad-Iraq from February 2014 to February 2015. Only 72 throat swab samples showed bacterial growth. The isolates were identified using Vitek 2 Compact system for Gram-Positive. Antibiotics susceptibility was examined using the BioMérieux Vitek2 compact system AST card. For direct molecular identification of *S. pyogenes*, *16S rRNA* and *16S-23S rRNA* gene amplification were used. Molecular screening for erythromycin resistance genes *erm(A), erm(B)* and *mef(A)* were done using PCR. The results of identification using Vitek2 GP show that 21 (29.2%) samples were *S. pyogenes*-positive while 51(70.8%) of samples were due to other causes of tonsillo-pharyngitis. The results of molecular identification of *S. pyogenes* strains using *16S rRNA* and *16S-23S rRNA* amplification showed that only four strains were positive for *16S-23S rRNA*, while two strains out of four were also positive for *16S rRNA*. According to the results of antibiotic sensitivity, there were seven strains resistant to erythromycin. The results of molecular screening for erythromycin resistant genes showed that all these resistant strains did not contain the resistant genes *erm(A), erm(B)* or *mef (A).* We conclude that, maybe there was a specific sequence variations in genes used for identification of *S. pyogenes*. Also, resistance to erythromycin could be attributed to causes other than the studied mutations, such as structural modification of erythromycin by phosphorylation, glycosylation or lactone ring cleavage by erythromycin esterase.**

Key words: *Streptococcus pyogenes*, molecular identification, erythromycin resistance genes.

INTRODUCTION

Streptococcus pyogenes is the leading cause of uncomplicated bacterial pharyngitis and tonsillitis commonly referred to as strep throat. It causes up to 15 to 30% of cases of acute pharyngitis that occurs in children in the age between 5 to 15 years. Other respiratory infections include sinusitis, otitis, and pneumonia. Also, it causes skin infections and post-streptococcal sequel, rheumatic fever, glomerulonephritis that may follow streptococcal diseases, and occur in 1 to 3% of untreated infections (Cunningham, 2000; Carapetis et al., 2005; Tart et al., 2007). Accurate diagnosis is essential for appropriate antibiotic selection. Penicillin should be a first choice of

antibiotics in acute tonsillitis while macrolides such as erythromycin is reserved for patients allergic to penicillin. Ketolides such as telithromycin have the activity against *S. pyogenes* which is resistance to erythromycin (Ben Zakour et al., 2012; Shulman et al., 2012). The mechanisms of action of erythromycin involve the inhibition of bacterial protein synthesis by binding reversibly to the subunit 50S of the bacterial ribosome, thereby inhibiting translocation of peptidyl-tRNA. The action is bacteriostatic, but can also be bactericidal in high concentrations (Giguere, 2013). Resistance to erythromycin in *S. pyogenes* can be caused by the following main mechanisms: 1) modification of the 23SrRNA by rRNA adenine-*N6*-methyltransferase encoded by horizontally acquired *erm* A and *erm* B (Jasir et al., 2000; Giovanetti et al., 2003; Albrich et al., 2004; Farrell et al., 2006; Brenciani et al., 2007); 2) active drug efflux via a trans-membrane pump encoded by horizontally acquired *mef*. This mechanism is mediated by a membrane-associated protein that pumps the antibiotic out of the cell, keeping intracellular concentrations low and preventing the binding of antibiotics to the ribosome (Nord et al., 2004; Del Grosso et al., 2011; Giovanetti et al., 2012; Giovanetti et al., 2003) mutations comprising a change in domain V of 23S rRNA as a result of a mutation in all the six copies of *rRNA* gene (Bingen et al., 2002). Additional mechanisms of erythromycin resistance include structural modification of erythromycin by phosphorylation (Davies and Davies, 2010), glycosylation (Hawkey and Jones, 2009), and lactone ring cleavage by erythromycin esterase (Levi and Marshal, 2004).

The *16S rRNA* and *23S rRNA* are targets for identification of microorganisms at the species, genus or family level. These genes contain both conserved regions and areas of variability sufficient for specific identification of bacteria. The ribosomal intergenic spacer region (ISR), a stretch of DNA that lies between the 16S rRNA and the 23S rRNA subunit genes, proved to be much more variable than the adjacent 16S and 23S ribosomal genes and this region can be used as method of differentiation of many species within genus and as method of identification of certain bacteria (Hassan et al., 2003). In this study, molecular identification of *S. pyogenes* using *16SrRNA* and *23SrRNA* and screening for erythromycin resistance genes was performed.

MATERIALS AND METHODS

Samples collection

One hundred and twenty five throat swab samples were collected from children between 2 to 12 years old with tonsillo-pharyngitis infections attended to by the AL-Imammain AL-Kadhimain Medical city-Baghdad-Iraq and Pediatric Caring Hospital-Baghdad-Iraq from February 2014 to February 2015. Throat swab samples were taken according to clinical evaluation recommendation of physicians (Vandepitte et al., 2003). Information from patient parent was taken including age, sex, duration of infection, previous treatment and stage of throat infection (acute or chronic). The study protocol was approved by The Ethical Committee of College of Medicine-Al Nahrain University.

Identification of *S. pyogenes*

Throat swab sample was cultured on blood agar plate that was incubated aerobically with 5 to 10 % CO_2 at 37°C for 18 to 24 h in a candle jar (Vandepitte et al., 2003). The blood agar plates were examined for morphology and cultural characteristic that include appearance of colonies and beta-hemolytic zone around colonies on blood agar plate. For purification, growing beta-hemolytic streptococci was inoculated on sodium azide media, which is consider as a selective agar used for the selective isolation of *S. pyogenes*. Also, catalase test, microscopical examination of Gram stain and Bacitracin sensitivity test were done (Vandepitte et al., 2003). The isolates were identified with Vitek 2 Compact system for Gram-Positive Identification, card 2GP (bioMérieux-France).

Antibiotics susceptibility assay

Minimal inhibitory concentration (MIC) and antibiotics susceptibility were examined using the BioMérieux Vitek2 compact system AST card (bioMérieux-France) according to manufacturer instructions. It is an automated colorimetric method used for identification of bacteria and for detection susceptibility of bacterial isolates against different type of antibiotics. A suspension of overnight pure culture of *S. pyogenes* was prepared by transferring sufficient quantity of bacterial colonies to 3 ml of sterile saline (0.45%NaCl). The turbidity was adjusted to (0.5-0.63) MacFarland turbidity range and measured using a turbidity meter. Then, the suspension was transferred to the apparatus which contain the card that loaded with 9 type of antibiotics included erythromycin, as indicated in Table 1. Measurement of MIC and sensitivity were done using optical system inside the apparatus and the result that was obtained after 18 h of incubation were computerized analyzed which referred to the MIC and whether this isolate had sensitivity, intermediate sensitivity or resistant to each antibiotic found in the card.

Identification of *S. pyogenes* strains using PCR

Genomic DNA was extracted from *S. pyogenes* strains using WIZARD Genomic DNA Extraction Kit (Promega, USA) following manufacture instructions. For direct molecular identification of *S. pyogenes*, *16S rRNA* and *16S-23S rRNA* were used. Two primer sets were used for molecular identification of *S. pyogenes* (Table 2) (Nandi et al., 2008). Briefly, two PCR master mixes (final volume 25 µl per reaction) were prepared, one for each gene as in the following: (final concentration per one réaction): 1XPCR buffer (Promega, USA), 200 µm dNTPs (Promega, USA), 100 pmol of each forward and reverse primers (Alpha, USA) and 1.25 U/reaction of GoTaq DNA polymerase (Promega, USA). Two microliters (equivalent to 100 ng) of DNA was added for each reaction tube, except the no template control tube (NTC). PCR

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Table 1. Vitek2 GP susceptibility cards contents.

Antibiotic	Concentrations µg/ml	Calling range	
		≤	≥
Ampicillin	0.5,1,4,8	0.25	16
Benzylpenicillin	0.06,0.12,0.5,2	0.06	8
Cefotaxime	0.25,0.5,1,2	0.12	8
Ceftriaxone	0.12,0.25,1,4	0.12	8
Clindamycin	0.12,0.25,0.5	0.25	1
Erythromycin	1,2,4,16	0.12	8
Levofloxacin	1,2,4,16	0.25	16
Tetracycline	0.12,0.25,1,4	0.25	16
Trimetheprim/ Sulphamethaxazole	8/152,16/304,64/1216	10	320

Table 2. Primer sequences and molecular size used in molecular identification of *S. pyogenes*.

Gene	Sequence of forward primer	Sequence of reverse primer	Product size (bp)
16S rRNA (A)	5'AAGAGTTTGATCCTGGCTCAG3'	5'GGTTACCTTGTTACGACTT3'	1500
16-23S rRNA (B)	5'TTGTACACACCGCCCGTCA3'	5'GGTACCTTAGATGTTRCAGTTC3'	800

Table 3. Primer sequences and molecular size used in erythromycin resistance genes.

Gene	Sequence of forward primer (5´- 3´)	Sequence of reverse primer (5´- 3´)	Product size (bp)
erm (A)	AGAAGGTTATAATGAAACAGA	GGCATGACATAAACCTTCAT	260
erm(B)	GAAAAGGTACTCAACCAAATA	AGTAACGGTACTTAAATTGTTTAC	640
mef(A)	AGTATCATTAATCACTAGTGC	TTCTTCTGGTACTAAAAGTGG	350

reaction tubes were transferred into thermal cycler (eppendrof, Germany) that was programmed as following: 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 49°C for Gene A and 55°C for Gene B for 2 min, 72°C for 2 min. Final extension was done at 72°C for 10 min. The selection of optimum annealing temperature came after multiple optimization experiments. PCR products (10 µl from each) were resolved by 1% agarose gel electrophoresis.

Molecular screening for erythromycin–résistant gènes in *S. pyogenes*

The sequence of oligonucleotide primers sets used in PCR reactions to amplify resistance genes *erm(A), erm(B)* and *mef(A)* are shown in Table 3. PCR reaction was done according to Morosini et al. (2003). Optimization for annealing temperature was done at 52°C.

Statistical analysis

Data were analyzed using SPSS version 16 and Microsoft Office Excel 2007. Nominal data were expressed as number and percent. Fischer Exact test was used for comparison of frequency. P-value less than 0.05 were considered significant.

RESULTS

Isolation and identification of *S. pyogenes*

This study was carried out in 125 throat swabs isolated from throat of children from 2 to 12 years old. Fifty three of total samples were excluded from this study because no bacteria were isolated. The remaining 72 samples were identified using bacteriological test. Also, commercial Vitek2GP identification card was used and the identifications probabilities were ranged from 86 to 99%. The results obtained by using Vitek2 GP identification system showed that 21 (29.2%) samples were *S. pyogenes* positive and 51(70.8%) samples were due to other causes of tonsillo-pharyngitis as shown in Table 4. The results of molecular identification of *S. pyogenes* strains using *16SrRNA* and *16S-23SrRNA* showed that only four strains were positive for *16S -23SrRNA*, while two strains out of four were also positive for*16S rRNA*, as seen in Figure 1.

Distribution of streptococci throat infection in disease phases acute and chronic *tonsillopharyngitis*

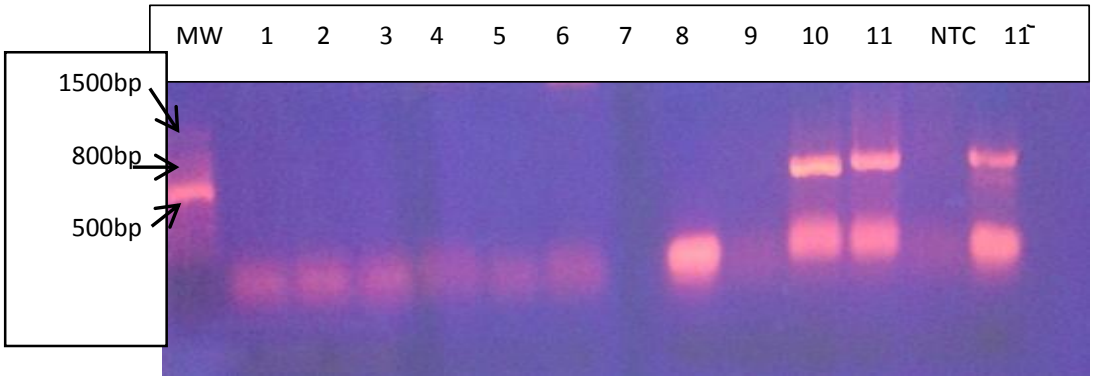
In this study, the number of isolates from acute cases were 5 isolates which represent (23.81%) of total cases while from chronic cases were 16 isolates which represent (76.19%) of total cases.

Antimicrobial susceptibility patterns of *S. pyogenes*

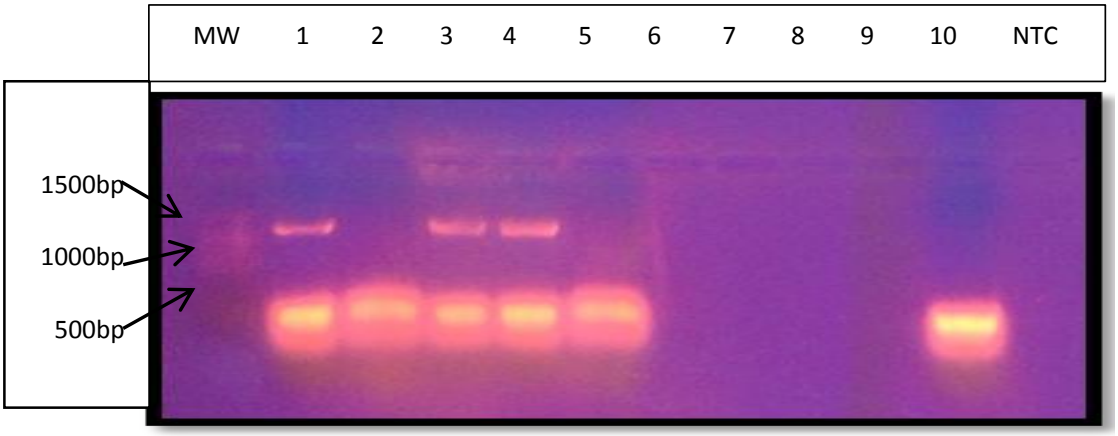
The antimicrobial susceptibility patterns of *S. pyogenes*

Table 4. Type and number of bacterial strains isolated from children with tonsillopharyngitis by using Vitek identification kit.

Throat swab bacteria isolated	Number of strains	Percentage (%)
Streptococcus pyogenes	21	29.2
Streptococcus agalactiae	2	2.8
Streptococcus pneumonia	4	5.6
Streptococcus mitis	2	2.8
Streptococcus parasanguinis	2	2.8
Staphylococcus aureus	41	56.8
Total	72	100



(A)



(B)

Figure 1. Agarose gel electrophoresis of amplified products of identification genes of *S. pyogenes*. **(A)** Agarose gel electrophoresis of amplified products of *16-23SrRNA* of *S. pyogenes*. Lane MW, molecular weight ladder of 100 bp. lane 1, 2, 3 to 9: negative results of *16-23S rRNA* (800 bp); lane 10, 11 and 11ˆ (as double): amplified products of *16-23S rRNA* (800 bp); lane NTC, no template control. **(B)** Agarose gel electrophoresis of amplified products of *16S rRNA* of *S. pyogenes*; lane MW, molecular weight ladder of 100 bp; lane 1, 3, 4: amplified products of *16SrDNA* (1500 bp); lane 2, 5 to 10, negative results of *16SrRNA* (1500 bp); lane NTC: no template control.

Table 5. The percentage of sensitivity patterns of *S. pyogenes* isolates against different types of antibiotics.

Antibiotic	Sensitive		Resistant		Intermediate	
	Number	%	Number	%	Number	%
Benzyl Penicillin	21	100	0	0	0	0
Ampicillin	21	100	0	0	0	0
Cefotaxime	8	38.09	13	61.90	0	0
Ceftriaxone	8	38.09	13	61.90	0	0
Levofloxacine	20	95.23	0	0	1	4.76
Erythromycin	11	52.38	7	33.33	3	14.28
Clindamycin	15	71.43	6	28.57	0	0
Tetracycline	10	47.62	11	52.38	0	0
TMS	21	100	0	0	0	0

strains against various types of antibiotics according to Vitek 2 system were shown in Table 5.

Molecular screening for erythromycin resistant genes in *S. pyogenes*

According to the results of antibiotic sensitivity obtained by using Vitek2 AST system, there were seven strains resistant to erythromycin. The results of molecular screening for erythromycin resistant genes showed that none of these resistant strains have the resistant genes *erm(A)*, *erm(B) or mef (A).*

Telithromycin activity against erythromycin resistant strains of *S. pyogenes*

The result of sensitivity test of telithromycin disc against erythromycin- resistant strains of *S. pyogenes* showed that 6 out of 7 strains have full sensitivity to Telithromycin disc with MIC value of ≤0.5 µg/ml while the remaining resistant strain showed intermediate sensitivity against this antibiotic with MIC value range from 1 to 2 µgm/ml.

DISCUSSION

Identification of *S. pyogenes*

The important cause of the tonsillitis is bacterial and viral causes and about 30 to 40% of bacterial tonsillitis cases are caused by *S. pyogenes* (Abd Al-Kareem et al., 2004). Result of this study showed that from 72 patients with tonsillo-pharyngitis, 21 strains indicated the presence of *S. pyogenes*, 2 strains of *Streptococcus agalactiae,* 4 strain of *Streptococcus pneumonia,* 2 strains of *Streptococcus mitis,* 2 strains of *Streptococcus parasanguinis* and 41 strain of *Staphylococcus aureus*. Kurien et al. (2000) and Wessels (2011) showed that the most common bacterial pathogens in the upper respiratory tract infection were *S. pyogenes* and *S. aureus*. In addition to identification by using Vitek2, *S. pyogenes* was identified using *16S rRNA* and *16S-23S rRNA*. The result showed that 4 strain out of 21 carried *16S-23S rRNA*, 2 out of these 4 strains additionally carried *16S rRNA*. The absence of identification genes in the remaining *S. pyogenes* strains may be due to the genetic variations. It was refereed to that the absence of intra-species genetic variation at *16S rRNA* subunit but documented variation in inter-genic *16S-23S* spacer region (Clarridge, 2004; Petti et al., 2005; Nandi et al., 2008; Lal et al., 2011).

In this study, the percentage of *S. pyogenes* that caused chronic tonsillopharyngitis was 76.19%; this result was significantly higher than the results of Afaf et al. (2004) in Egypt who found that (18.5%) of the *S. pyogenes* strains were responsible for chronic tonsillopharyngitis. The low incidence of streptococcal tonsillopharyngitis in present study may be due to the relatively small number of the throat samples collected from patient with tonsillo-pharyngitis.

Antibiotic susceptibility patterns of the *S. pyogenes* using Vitek2 AST system

In this study, the susceptibility test of *S. pyogenes* strains using Vitek2 AST showed that all *S. pyogenes* strains were susceptible to penicillin group and penicillin remain the drug of choice for treatment of streptococcal pharyngitis, because the circumstances favorable for the development of resistance have not yet occurred, because this antibiotic is out of use in clinical practice in Iraq nowadays and the preference of the newest antibacterial drugs for prescription, as well as inefficient mechanisms for genetic transfer or barriers to DNA uptake and replication and β-Lactamase may not be expressed or may be potentially toxic to *S. pyogenes* (Malhotra-Kumar et al., 2005; Ramalhinho et al., 2012;

Rubio-Lopez et al., 2012). Also, it could be that PBPs of *S. pyogenes* contain no lengthy regions of similarity with genes from other streptococci, making it unlikely that the acquisition of penicillin resistance arises by homologous recombination with genes from other species (Ferretti et al., 2001). This study shows that the susceptibility of strains to cefotaxime and ceftriaxone was 38.09% which is due to the extensive and random prescribing of these antibiotics before doing culture and sensitivity test, as well as those antibiotics in Iraq are supplied as over counter medicines in private pharmacies against the regulations. The results obtained by Young et al. (2004) in South Korea, Oliver et al. (2007) in Spain and Huang et al. (2014) in Taiwan, shows that the susceptibility of *S. pyogenes* to cefotaxime and ceftriaxone was 100%.

In patients who are allergic to penicillin, macrolides such erythromycin and lincosamides such as clindamycin are alternative treatment choices (Shulman et al., 2012). In this study, the percentages of resistance of clindamycin and erythromycin for *S. pyogenes* were 28.75 and 33.33%, respectively. Huang et al. (2014) showed that the resistance to clindamycin and erythromycin were 2.1 and 16.4%, respectively. Other studies referred to low percentages of erythromycin resistance such as in America (8.6%), Asia-pacific region (10.9%), Europe (9.7%) and Latin America (2.7%) (Gordon et al., 2002). Shibl (2005) in Saudia Arabia showed that the resistant was only 6.3%, and similar percentages (4 to 10%) have been reported in Germany, UK, Portugal, Greece and Canada. The level of erythromycin resistance among strains was low which may be related to the low consumption of macrolides in these regions, or may be due to the absence of clonal spread of erythromycin-resistant strains (Silva-Costa et al*.,* 2012). The high percentage that is found in the current study could be attributed to high misuse of antibiotic in Iraq.

Screening for erythromycin resistant gene

Increases in macrolide resistance have been reported and the rapidly growing problem of antibiotic resistant *S. pyogenes* is increasing (Ray et al., 2010). In Iraq, information regarding the screening for erythromycin resistant genes of *S. pyogenes* strains was largely loosing. In this study, there were no strains related to *S. pyogenes* carrying the resistant genes. Bingen et al. (2000) referred that the predominance of a particular resistance genotype among macrolide-resistant strains were *mefA* in Spain (97% of 437 strains), Belgium (84% of 131 strains), Germany (56% of 54 strains) and Canada (92% of 72 strains) and *ermB* in France (55% of 93 strains). Richter et al. (2005) refereed to that of the population of macrolide-resistant *S. pyogenes* strains in the United States comprises similar proportions of strains containing *mefA* (43%) and *ermA* (46%), with a smaller fraction of strains having *ermB* (8.5%) and considerable variation among regions. A study by Dundar et al. (2010) in Turkey show that of 3 of 11 erythromycin resistant strains of *S. pyogenes* did not have *erm(A), erm(B),* and *mef(A)* and this may be due to the ribosomal mutations.

Effect of telithromycin on erythromycin resistant *S. pyogenes*

In this study, 6 out of the 7 erythromycin resistant strains had high sensitivity to telithromycin with MIC value ≤ 0.5 µg/ml while the remaining strain show intermediate resistance with MIC value range between 1 to 2 µg/ml. Such results may justify the effectiveness of this antibiotic as alternative of erythromycin in the treatment of streptococcal pharyngitis (Camara et al., 2013). Telithromycin was more active than 14 and 15 membered ring macrolides (azithromycin and clarithromycin) against erythromycin resistant *S. pyogenes* strains (Jalava et al., 2001). Telithromycin show good activity against clinical *S. pyogenes* isolates including erythromycin A-resistant strains harboring the *erm*(A) or *mef*(A) (efflux) genotype.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Antifungal, acute toxicity and mutagenicity activity of extracts from *Datura stramonium*, *Jacquinia macrocarpa* and *Krameria erecta* on *Fusarium verticillioides*

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The effect of *Baccharis glutinosa*, *Jacquinia macrocarpa*, and *Krameria erecta* extracts was investigated on the growth and the spore germination of *Fusarium verticillioides* (ATCC 52539). Brine shrimp (*Artemia salina*) was used to evaluate the potential acute toxicity of the fractions obtained from plant extracts. The butanol fraction of *J. macrocarpa* totally inhibited the radial growth for 144 h and up to 95% after 168 h. The ethyl acetate fraction of *B. glutinosa* caused 100% of radial growth inhibition for 96 h. The ethyl acetate fractions of *B. glutinosa* and *K. erecta* caused the higher inhibitory effect on *F. verticillioides* spore germination, 100 and 95%, respectively. All plant fractions tested at a concentration of 5.0 mg mL^{-1} caused 100% brine shrimp lethality after 24 h. The Ames test did not reveal the presence of an evident mutagenic activity.

Key words: Antifungal activity, plant extracts, brine shrimp bioassay, mutagenicity assay, *Fusarium verticillioides.*

INTRODUCTION

The plant species in Mexico are more than 26,000 from which 4,000 are estimated to have medicinal use (Mittermeier and Goettsch, 1992). In addition, some of them have exhibited other properties such as antifungal activity and might be considered natural bioactive substances for the control of post-harvest fungal infections. Plant extracts are generally assumed to be more acceptable and less hazardous than synthetic compounds and they might represent an alternative anti-fungal approach (Jobling, 2000).

Baccharis glutinosa Pers (syn.: *Baccharis salicifolia* (Ruiz & Pav.) Pers) and *Jacquinia macrocarpa* (syn.: *Jacquinia aurantica*), are traditional medicinal plants that belong to the *Asteraceae* and *Theophrastaceae* families, respectively (Barrows, 1967; Moreno-Salazar et al., 2008). Ethnic groups from Northwest Mexico have been

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using *B. glutinosa* for gastrointestinal disorders whereas they have used *J. macrocarpa* to prepare a mustard-colored dye from the fruits and a tea out of the flowers that strengthens the heart (Yetman and Van Devender, 2002). Also, cytotoxic and anti-inflamatory properties have been reported for *B. glutinosa* extracts (Fukuda et al., 2006; Abad et al., 2006; Abad and Bermejo, 2007). These plants are widely distributed from Southwest U.S.A. to central Mexico (Barrows, 1967; Moreno-Salazar et al., 2008). *Jacquinia* is native from West Indies where it is known as *J. aurantica*. DiSalvo (1974) reported that *B. glutinosa* aqueous extract of dried powdered leaves to inhibit dermatophytes *in vitro*. He mentioned that *B. glutinosa* is recommended in the southwestern desert of the United Sates for the therapy of athlete's foot caused by *Tinea pedis*. In addition, *Datura stramonium* has been reported to have antifungal activity.

Fractions from methanolic extracts of these plants have shown antifungal properties against some phytopathogenic and toxigenic molds such as *Aspergillus flavus*, *Aspergillus parasiticus* and *Fusarium verticillioides* (Buitimea-Cantúa et al., 2013). Those authors reported radial growth inhibition, hyphal diameter and length, and mycotoxin production. Based on the above, the aim of this study was to evaluate the antifungal properties of fractions of extracts obtained from *Krameria erecta*, *Baccharis glutinosa,* and *J. macrocarpa* on *F. verticillioides,* and to evaluate their mutagenic potential and acute toxicity.

MATERIALS AND METHODS

Plant materials

J. macrocarpa Cav. spp. pungens and *Krameria erecta* Willd ex Schult were collected in the area of Los Arrieros, Sonora (Latitude N 28° 20.538' W 111° 08.911' altitude 280 feet and latitude N 28° 19.526' W 111° 08.828' altitude 227 feet) during august 2010. Aerial parts of *B. glutinosa* Pers. were collected during February 2011 in the riverside of Tecoripa River near the rural community of Tecoripa, Sonora. A voucher sample of each plant was deposited at the Herbarium of the Scientific Research and Technology Department of the University of Sonora (DICTUS) in Hermosillo, Sonora (Mexico) to confirm its identification. The plant specimens were sealed in plastic bags, and transported to the laboratory.

Preparation of antifungal extracts

Plants were sun dried (35-40°C) for 2 weeks and milled (Pulvex 200, U.S.A.) to a particle size of 0.5-1.0 mm. Sixty grams of powdered aerial parts of each plant were extracted with 940 ml of 70% methanol by agitation for 1 h with a wrist action Burrel shaker (Burrel Corporation, Pittsburg, PA), and stored at 25°C for 3 days at darkness. The extracts were filtered first through Whatman filter paper No. 1 and then through micropore glass filter. The methanolic extracts (crude extracts) were evaporated to dryness at 40°C with vacuum in a Yamato rotary evaporator RE 300). Crude extracts were evaluated for antifungal activity. The extracts that showed the highest inhibition activity was evaporated to dryness and subjected to fractionation. Twenty grams of dried extracts were suspended in 1 L of water and sequentially partitioned with hexane, ethyl acetate, and n-butanol (Koketsu et al., 1996) and all were tested for antifungal activity. Plates with potato dextrose agar medium, PDA, (DIFCO, USA) were prepared using 5 mg mL^{-1} of each fraction [Ethyl acetate fraction of *B. glutinosa* and *K. erecta* (FAe *Bg*) and (FAe *Ke*), respectively and n-butanol fraction of *J. macrocarpa* (FB *Jm*)]. Petri dishes containing PDA prepared with each of the different solvents used for fractionation were used as controls.

Antifungal activity assay

A strain of *F. verticillioides* (ATCC 52539) was selected for its high fumonisin production. Fungal strain was activated in PDA and incubated at 25 ± 2°C for 10 days. Spores were harvested by pouring a sterile solution of 0.1% (v/v) Tween 80 into the flask and stirring the suspension with a sterile magnetic bar for 5 min. Spore concentration of the suspension was determined using a Neubauer chamber and adjusted to a final concentration of 1 x 10^5 spores/mL. Petri dishes containing PDA medium prepared with 5 mg mL^{-1} of plant extract fractions were centrally point-inoculated with 1 x 10^5 spores/mL and incubated in the darkness at 25°C for 7 days. Two types of controls were prepared, one contained PDA medium plus aliquots of each solvent and the other one containing only PDA media. Colony diameters were measured every 24 h using a caliper and compared to those grown in the control media until the fungal growth in the control reached the plate border. All the measurements were carried out by triplicate. The radial growth inhibition percentage was calculated using the following equation: Radial Inhibition (%) = [(Rc-Ri)/Rc] x 100. Where *Rc* is the mean value of the colony radius in the control media and *Ri* is the colony radius value of the colonies grown in PDA amended with the partitioned extracts.

Germination of spores

Petri dishes containing PDA amended with 5 mg mL^{-1} of extract fractions (FAe *Bg*, FAe *Ke,* and FB *Jm*) were inoculated by spreading 3 µl of a spore suspension containing 1x10^4 spores/mL and incubated at 25°C using a 12 h light/dark cycle (Precision Low temperature Illuminated Incubator 818, U.S.A.). Two types of controls were prepared, one contained PDA medium plus aliquots of each solvent and the other one contained only PDA media. Samples were taken every 4 h of incubation time and 200 spores were counted at random (germinated and non-germinated) using light microscope. Count of spores was performed until the control reached 100% of spores germinated. The number of germinated spores per plate was determined. A spore was considered germinated when the length of its germinal tube reached one-half of the spore diameter. Each germination experiment was made by triplicate. The inhibition of spore germination was determined using Equation 1, in which S_i represents the percentage of germinated spores in the plates treated with the extract fraction, and S_c was the percentage of germinated spores in the control containing each of the solvents (Paul et al., 1993).

$$\text{Inhibition (\%)} = \frac{\%S_c - \%S_t}{\%S_c} \times 100$$

Brine shrimp bioassay

In order to evaluate the potential acute toxicity of the fractions obtained from plant extracts, brine shrimp larvae assay was used (Jiménez et al., 1997). Dried *Artemia salina* eggs (0.1 g) were

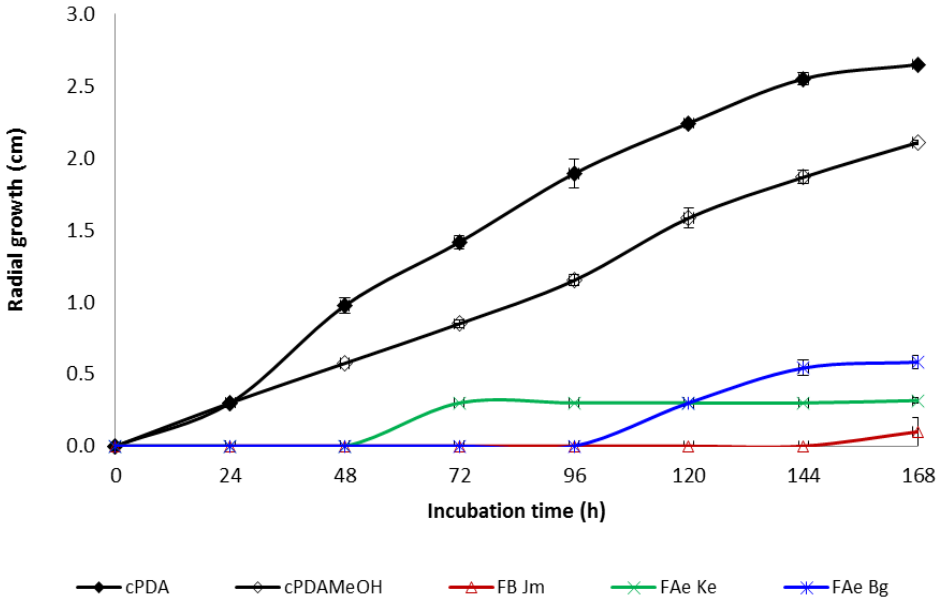

Figure 1. Radial growth of *Fusarium verticillioides* in PDA amended with 5 mg mL^{-1} of extracts fractions from *Jacquinia macrocarpa* (FB *Jm*), *Krameria erecta* (FAe *Ke*), and *Baccharis glutinosa* (FAe *Bg*).

deposited in 1 L of sterile marine water with aeration and light during 24 h to hatch. Brine shrimp larvae were exposed to 5.0, 0.5, 0.005, and 0.0005 mg mL^{-1} of the extract fractions for 24, 48, and 72 h. The number of dead larvae was recorded every 24 h to estimate the percentage of mortality. The assay was carried out by quintuplicate.

Mutagenicity assay

The mutagenic potential of the fractions obtained from plant extracts was determined according to the procedure described by Maron and Ames (1983) using *Salmonella* tester strains TA98 and TA100, with and without bioactivation (S9). Each was placed on nutrient broth (Difco Nutrient Broth) for reproduction during 12 h at 37°C in a circulation water bath at dark. One-hundred microliters of partitioned plant extracts, FAe *Bg*, FAe *Ke* and FB *Jm*, were deposited in test tubes (5, 0.5, 0.005, 0.0005, 0.00005, and 0.00005 mg mL^{-1}). Then, each tube was combined with 2.0 mL of bacteriologic agar (Sigma Chemical Co.) supplemented with histidin and biotin, 100 µL of bacterial culture, and 500 µL of S9 mix. This mixture was poured onto minimal glucose agar Petri dishes and incubated for 48 h at 37°C. For mutagenicity, positive control sodium azide was used (without S9) and aflatoxin B1 (with S9). The number of revertants was counted using a colony counter and compared against the controls. The assay was carried out by triplicate.

A completely randomized design of the radial growth and spore germination was carried out. The JMP 2004 software computed the analysis of variance and the means were compared with the Tukey multiple range tests ($P<0.05$) (JMP vs. 5.0, SAS Institute Inc., USA).

RESULTS AND DISCUSSION

The extract fractions exhibited a moderate to high antifungal activity against *F. verticillioides*. No fungicide effect was observed, only an inhibitory activity was detected in the fungus growth when compared to controls. Controls with solvents and pure PDA control showed the higher radial growth. When inoculated on PDA containing the extracts fractions, the radial growth of the fungi was delayed during the incubation time (Figure 1). The BF *Jm* totally inhibited the radial growth for 144 h and up to 95% after 168 h (Table 1). On the other hand, the FAe *Bg* caused 100% of radial growth inhibition for 96 h, after that the inhibitory effect was reduced to 72%. Treatment with FAe *Ke* also inhibited the radial growth in 100% for 48 h and 65% after 72 h of incubation. These results are in agreements with a previous work (Rosas-Burgos et al., 2009), which reported an inhibition of 67% of *F. verticillioides* radial growth. This result is of relevance for the present study because it confirms that *Baccharis glutinosa* has a fungistatic activity; plant specimens used in the present study were collected in a different year to those used by Rosas-Burgos et al. (2009) and similar results were reached. This might suggest that bioactive compounds are present in *B. glutinosa* independently of the year in which the plant is collected. Nevertheless, a recent study showed that concentration of total phenolic compounds and flavonoids on *B. dentata* showed significant seasonal variation (Sartor et al., 2013). Regarding the chemical constituents found in the genus, coumarins, flavonoids and terpenoids are the most frequently reported (Cifuente et al., 2002; Simoes-Pires et al., 2005; Wachter et al., 1999). On other hand, Kurdelas et al. (2010) isolated three coumarins from *Baccharis darwinii*. The effect of extracts may be

Table 1. Radial growth inhibition (%) of *Fusarium verticillioides* in PDA amended with 5 mg mL^{-1} of extracts fractions from *Jacquinia macrocarpa* (FB *Jm*), *Krameria erecta* (FAe *Ke*), and *Baccharis glutinosa* (FAe *Bg*).

Incubation time (h)	FB Jm	FAe Ke	Fae Bg
48	100 ± 0.0	100 ± 0.0	100 ± 0.0
72	100 ± 0.0	65 ± 0.0	100 ± 0.0
96	100 ± 0.0	74 ± 0.0	100 ± 0.0
120	100 ± 0.0	81 ± 0.0	81 ± 0.0
144	100 ± 0.0	84 ± 0.0	71 ± 5.1
168	95 ± 8.2	85 ± 1.4	72 ± 3.6

FB Jm = n-Butanol fraction of *J. macrocarpa*; FAe Ke = ethyl acetate fraction of *K. erecta*; FAe Bg = ethyl acetate fraction of *B. glutinosa*.

Table 2. *Fusarium verticillioides* spore germination rate on PDA with and without the evaluated extracts fraction (5 mg mL^{-1}).

Treatment	Spore germination rate (% EG h^{-1})	Radial extension rate (cm h^{-1})
FAe *Bg*	0.000	0.0113
FAe *Ke*	0.354	0.0125
FB *Jm*	1.438	0.0003
cPDAMeOH	11.406	0.0129
cPDA	11.750	0.0185

FAe *Bg* = *Baccharis glutinosa* ethyl acetate fraction; FAe *Ke* = *Krameria erecta* ethyl acetate fraction; FB *Jm* = *Jacquinia macrocarpa* n-butanol fraction; cPDAMeOH = PDA plus methanol control; cPDA = Control of PDA; EG h^{-1} = Germinated spores.

due to their chemical composition and probably to the membrane composition of the fungi. *B. dracunculifolia* DC, a native plant of South America, is one of the most studied of this genus and baccharin (3-prenyl-4-(dihydro-cinnamoyloxy) cinnamic acid) is the chemical compound isolated from its aerial parts. Tabti et al. (2014) mentioned that terpene hydrocarbons and phenolic compounds affects the fungi development. Information on the mechanism(s) of action by these type of compounds in *Baccharis* is not available. Velluti et al. (2005) mentioned that other authors have attributed it not only to the presence of terpenes, phenolic compounds, and other components, but also to the chemical structure, such as the presence of hydroxyl groups in their phenolic compounds.

Also, the values of radial extension rate, determined from the slope of the radial growth versus time during the linear growth phase, were reduced (Table 2). The lower value corresponds to the treatment with FB *Jm* which caused 100% of inhibition. Result indicate that spores inoculated on control treatments began to germinate after 4 h and reached the 100% of germination at 14 h. Spores inoculated on media containing FAe *Bg* and FAe *Ke* caused 100 and 95% germination, respectively at 14 h. On the other hand, spores cultivated in the presence of FB *Jm* were poorly affected.

The fractions FAe *Bg* and FAe *Ke* caused higher inhibitory effect on *F. verticillioides* spore germination, 100 and 95%, respectively (Figure 2). FB *Jm* exerted the lowest effect on spore germination inhibiting only 19.0% and allowed the higher germination velocity compared to the other plant fractions (Figure 2). The first morphological change in spore germination is called swelling in which the diameter of the spore increases. It involves water uptake and a decrease in the micro-viscosity of the cytoplasm. Also, molecules are directed to the cell cortex to enable addition of new plasma membrane and cell wall (Bartnicki-Garcia and Lippman, 1977; Momany, 2002). At later stages of development, the growth speed of the germ tube increases and the functional organization of the hyphal tip area acquires its full potential. The structure of the fungal cell wall is unique to the fungi and it is composed of chitin, glucans, mannans and glycoproteins (Bowman and Free, 2006). Damage on the fungal cell wall produces morphological alterations, inhibition of fungal growth or apoptotic cell death (Escalante et al., 2008; Alonso et al., 2010; Khan and Nasreen, 2010), which are presumably the result of alterations caused to the components of the cell wall, β-glucan and chitin. Recent research has shown that extracts from *B. glutinosa* and *J. macrocarpa* have chitinase activity against polymeric extracts from *A. flavus* and *F. verticillioides* (Buitimea-Cantúa et al., 2013), which helps to explain our findings.

Table 3 shows the data obtained from the negative control of *A. salina* suspended in marine water. *A. salina* exposed to sodium azide (5.0 and 0.5 mg mL^{-1}, positive control) reached 100% mortality after 24 h. All plant fractions tested at a concentration of 5.0 mg mL^{-1} caused 100% brine shrimp lethality after 24 h. The extract fraction from *J. macrocarpa* (BF *Jm*) showed similar toxicity effects than sodium azide after 24 h when *A. salina* was either exposed to 5.0 or 0.5 mg mL^{-1}. However, when BF *Jm* concentration decreased, the brine shrimp mortality also diminished. This plant extract fraction caused the lowest mortality from the three plant extracts evaluated. The other two extracts showed similar mortality at all of the concentrations evaluated. Our results suggest the presence of toxic compounds in each of the plant extracts, which are able to physiologically affect *A. salina*.

The positive mutagenicity controls, sodium azide and aflatoxin B1, tested in *Salmonella* Thyphimurim strains TA 98 and TA 100, are presented in Table 4. We observed that they were sensitive in this experiment and reproducible results could be achieved. Plant extracts did not induced any mutagenic effect on both *Salmonella* tester strains (Table 5). Mutagenicity exerted by extracts was considered negative since the number of revertant

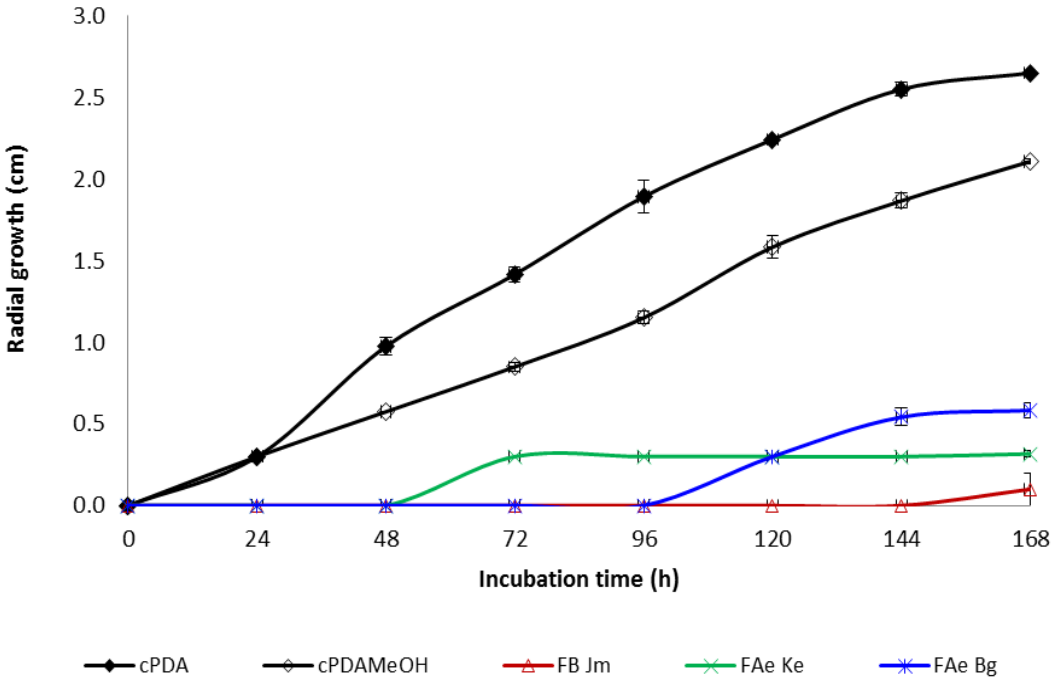

Figure 2. Radial growth of *Fusarium verticillioides* in PDA amended with 5 mg mL^{-1} of extracts fractions from *Jacquinia macrocarpa* (FB *Jm*), *Krameria erecta* (FAe *Ke*), and *Baccharis glutinosa* (FAe *Bg*).

Table 3. Mortality of *Artemia salina* when exposed to different treatments

Treatment (mg mL^{-1})	Exposition time (h)		
	24	48	72
Marine water with sodium azide	1.67 ± 1.7^{a}	4.71 ± 4.7^{a}	84 ± 5^{a}
0.005	4 ± 4^{a}	36 ± 12^{b}	84 ± 2^{a}
0.05	24 ± 6^{b}	58 ± 13^{c}	93 ± 5^{b}
0.5	100 ± 0^{c}	100 ± 0^{f}	100 ± 0^{c}
5.0	100 ± 0^{c}	100 ± 0^{f}	100 ± 0^{c}
FB *Jmb*			
0.005	26 ± 4^{b}	55.6 ± 12^{bc}	92.71 ± 3^{b}
0.05	55 ± 10^{c}	70 ± 10^{d}	97 ± 2b^{c}
0.5	99 ± 0.8^{e}	100 ± 0^{f}	100 ± 0^{c}
5.0	100 ± 0^{e}	100 ± 0^{f}	100 ± 0^{c}
FAe *Ke*			
0.005	54.7 ± 10^{c}	86 ± 5^{e}	97 ± 2^{b}
0.05	88 ± 8.5^{d}	96 ± 4^{f}	100 ± 0^{c}
0.5	81.1 ± 6.8^{d}	100 ± 0^{f}	100 ± 0^{c}
5.0	100 ± 0^{e}	100 ± 0^{f}	100 ± 0
FAe Bg			
0.005	49.1 ± 14^{c}	89 ± 4.6^{e}	99.2 ± 0.8^{c}
0.05	60.2 ± 12^{c}	95.5 ± 2^{ef}	100 ± 0^{c}
0.5	87.5 ± 5^{d}	92.8 ± 3^{f}	100 ± 0^{c}
5.0	100 ± 0^{e}	100 ± 0^{f}	100 ± 0^{c}

FB *Jm* = *Jacquinia macrocarpa* n-butanol fraction; FAe *Ke* = *Krameria erecta* ethyl acetate fraction; FAe *Bg* = *Baccharis glutinosa* ethyl acetate fraction.

Table 4. *Salmonella* test strains TA98 and TA100, with and without bioactivation (S9) exposed to different concentrations of mutagenicity positive controls.

Concentration (mg mL^{-1})	TA 98			TA 100		
	RI	RE	RM	RI	RE	RM
Sodium azide without S9						
0.015	922 ± 65^{b}	26	35	2140 ± 111.4	224	10
0.15	1147 ± 117^{c}	26	44	2388 ± 67.7	224	11
1.5	2053 ±88^{d}	26	79	3474 ± 317.0	224	15
15	253 ±42^{a}	26	10	1626 ± 213.0	224	7
Aflatoxin B1 with S9						
5	41.0 ± 7^{a}	26	1.58	236 ± 12.5	224	1.16
50	42.0 ± 3^{a}	26	1.63	278 ±15.6	224	1.36
500	932.0 ± 46^{c}	26	35.86	2867 ± 415.0	224	14.1
5000	205.0 ±9^{b}	26	7.88	329 ± 66.3	224	1.6

RE = Spontaneus revertants; RI = induced revertants; RM = mutagenicity ratio.

Table 5. Antimutagenic potential of the plant extracts fractions. Both *Salmonella* test strains TA98 and TA100, with and without bioactivation (S9), were exposed to different concentrations of the extracts fractions.

Concentration (mg mL^{-1})	*Bg* FAe TA 100	*Bg* FAe TA 98	*Jm* FB TA 100	*Jm* FB TA 98	*Ke* FAe TA 100	Ke FAe TA 98
With S9						
0	231.5 ± 10.6	34.5 ± 12.0	162.5 ± 48.8	47.5 ± 9.2	187 ± 83.4	45.5 ± 12.0
0.00005	201.5 ± 17.7	33 ± 4.2	229 ± 82.3	49 ± 11.3	272.5 ± 13.4	36 ± 5.7
0.0005	217 ± 42.4	44.5 ± 12.0	221 ± 77.8	53 ± 8.5	276 ± 9.9	36 ± 0
0.005	271.5 ± 10.6	40 ± 4.2	258.5 ± 51.6	43 ± 4.2	261.5 ± 37.5	34.5 ± 4.9
0.05	245.5 ± 7.8	36 ± 4.2	257.5 ± 21.9	34 ± 1.4	271.5 ± 50.2	30.5 ± 6.4
0.5	241.5 ± 24.7	38 ± 1.4	277 ± 15.6	38.5 ± 7.8	295 ± 46.7	53 ± 29.7
5	191 ± 48.1	41.5 ± 2.1	278.5 ± 16.3	42 ± 21.2	210 ± 53.7	34 ± 41.0
Without S9						
0	231.5 ± 10.6	34.5 ± 12.0	162.5 ± 48.8	47.5 ± 9.2	187 ± 83.4	45.5 ± 1.0
0.00005	212 ± 4.2	35 ± 14.1	242 ± 15.6	43.5 ± 10.6	225 ± 2.8	40 ± 0
0.0005	214 ± 5.7	32 ± 5.6	248 ± 26.8	37 ± 1.4	256 ± 19.8	39 ± 9.8
0.005	225.5 ± 0.7	28 ± 0	237.5 ± 16.7	41 ± 1.4	194 ± 39.6	36 ± 9.8
0.05	250.5 ± 36.6	26 ± 2.8	240.5 ± 0,7	38 ± 4.24	204 ± 42.4	42 ± 19.8
0.5	231.5 ± 3.5	26.5 ± 0.7	242 ± 1.4	42 ± 0	229.5 ± 24.7	44.5 ± 9.2
5	192 ± 11.3	28.5 ± 3.5	266 ± 7.1	33 ± 1.4	230.5 ± 24.7	56 ± 12.7

All values represent mean of triplicate determination ± standard deviation.

per plate observed did not double the number of spontaneous revertants. Similar findings have been were reported (Nogueira et al., 2011); they found that fractions from *Baccharis trimera*, evaluated *in vivo* and *in vitro*, were not mutagenic. Also, other authors reported no genotoxic activity of *Baccharis incarum* on *Drosophila* melanogaster (Berzaín and Rodrigo, 2006). These findings suggest that the genus *Baccharis* might not be of potential toxicity to superior animal organisms; however, further investigation should be performed for a full toxicity assessment.

This study indicates that the plant extracts had anti-fungical activity on *F. verticillioides* and can be exploited in the future to reduce fungal spread. They delayed radial growth during the the incubation time. Ethyl acetate fractions of *B. glutinosa* and *K. erecta* caused the higher inhibitory effect on *F. verticillioides* spore germination, 100 and 95%, respectively. Suppresion on spore production could be the major contribution to limit the pathogen spread. Plant fractions tested at 5.0 mg mL^{-1} caused 100% brine shrimp lethality after 24 h and the Ames test did not reveal mutagenic activity.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Full Length Research Paper

Connecting DNA origami structures using the biotin-streptavidin specific binding

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This work made use of the strong interaction between biotin and streptavidin to connect designed DNA origami structures. The caDNAno software was used to design a 6 layer 3D origami cross-like structure. Selected DNA strands at the engineered attachment sites on the DNA origami structure were biotinylated. After folding of the origami structures, the biotinylated strands stick out of the attachment sites. Purified samples of origami structures were then mixed with streptavidin and the mixture purified. After characterization, we see that attachment only occurs at the biotinylated sites. Agarose gel electrophoresis, UV-vis spectroscopy and TEM were used to characterize the structure.

Key words: DNA origami, interaction, biotin-streptavidin, nanomaterials, TEM.

INTRODUCTION

The specific binding of bases is exploited to self-assemble DNA which gives a large amount of control over nanoscale devices assembly. Seeman (1982, 2003) laid down the theoretical model that allowed the use of DNA as a building material for the construction of devices at the nanoscale. DNA has the capacity to be programmed for self-assembly and has also a high stability making it possible to be used in device construction. There are a large number of materials ranging from metals, semiconductors to biological materials that can chemically be attached to DNA. Researchers have used DNA to construct a large number of composite structures (Chen and Seeman, 1991; Ekani-Nkodo et al., 2004; Fu and Seeman, 1993; Hou et al., 2005; Li et al., 1996; Liu et al., 1999; Winfree et al., 1998). The search continued to build miniaturized structures to design advanced materials with high performance. Rothemund (Rothemund, 2006) came out with the versatile, robust and significant DNA origami method which could be used to construct both 2-D and 3-D structures. The DNA origami method encompasses the folding of a long single-stranded scaffold DNA by shorter single-stranded staple DNA sequences. The mixture is then heated and annealed at room temperature for

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Abbreviations: EDTA, Ethylenediaminetetraacetic acid; **TEM,** Transmission electron microscopy.

several hours or days depending on whether single- or multi-layered structures are involved (Douglas et al., 2009a; Douglas et al., 2009b).

Rothemund was the first to demonstrate the functionalization of DNA origami surfaces (Rothemund, 2006). Since then other researchers have used varying arrays of nanomaterials to functionalize origami surfaces. This is made possible by the use of sticky ends which protrude on the surface. Nanomaterials which are functionalized with complementary sequences are then made to hybridize with these sticky ends. In so doing they attach themselves on the surface. The covalent bond between gold and sulfur is employed in the case of gold. Several groups have made use of this approach (Amoako et al., 2013; Ding et al., 2010; Maune et al., 2010, Pilo-Pais et al., 2011; Shen et al., 2012) to functionalize origami structures. Several groups (Jungmann et al., 2011; Lavella et al., 2012) have made use of the strong biotin-streptavidin interaction to functionalize DNA and DNA origami structures.

Selected staples are modified and extended with biotin making it possible for streptavidin binding with the DNA strand or DNA origami structures. Li et al. (2004) used this interaction to control the templating of two forms of triple crossover molecules through self-assembly. Lyonnais et al. (2008) also used the interaction to conjugate DNA and carbon nanotubes. Eskelinen (Eskelinen et al., 2011) and fellow workers have also used the biotin-streptavidin interaction to assemble carbon nanotubes on DNA origami. In order to reconfigure DNA origami pliers, Kuzuya (Kuzuya et al., 2011) and colleagues used the strong binding biotin-streptavidin interaction.

All these researchers made use of the biotin-streptavidin interaction to functionalize the DNA strand or DNA origami structures. In this work, we demonstrate the use of this interaction to connect two or more DNA origami structures. The square lattice caDNAno (Ke et al., 2009) software was used to design a six layered 3D origami cross-like structure. The connecting sites with DNA strands extended with biotin were designed at the two ends of the long axis of the cross-like origami structures. By means of streptavidin addition to the DNA origami solution, the origami structures are extended.

MATERIALS AND METHODS

Chemicals and supplies

Ethylenediaminetetraacetic acid (EDTA), utrapage purified DNA oligonucleotides, streptavidin and DNA oligonucleotides extended with biotin were purchased from Sangon Biotech (Shanghai, China) Co. Ltd. Tris(hydroxymethyl) aminomethane (Tris), Agarose M, and magnesium acetate tetrahydrate ($(CH_3COO)_2Mg{\cdot}4H_2O$) were obtained from Bio Basic Inc (Markham, Canada). NA-red, and 6X loading buffer were bought from Beyotime Institute of Biotechnology (Haimen, China). Wide range DNA marker was purchased from Takara Biotechnology (Dalian, China) Co. Ltd. The single-stranded viral genomic DNA M13mp18 used in the experiments was purchased from New England Biolabs (Ipswich, UK). We purchased boric acid (H_3BO_3), magnesium chloride ($MgCl_2$), and acetic acid ($C_2H_4O_2$) from Sinopharm Chemical Reagent (Shanghai, China) Co. Ltd. Freeze 'N' Squeeze DNA gel-extraction spin columns were bought from Bio-Rad Laboratories Inc. (Hercules, USA). Carbon copper grids and mica were purchased from Beijing Zhongjingkeyi Technology Co. (China) Ltd. and finally uranyl acetate ($UO_2(CH_3COO)_2{\cdot}2H_2O$) was purchased from Structure Probe, Inc. (Beijing, China).

Folding and purification of DNA origami cross-like structures

Our sample was prepared based on the procedures outlined in (Castro et al., 2011) with slight changes to the annealing process by combining 10 nM scaffold (M13mp18), 100 nM of each of the 178 staple oligonucleotides which were used without further purification, buffer and salts including 5 mM Tris, 1 mM EDTA (PH 7.9 at 20°C), and a magnesium screen covering 7 different concentrations from 12 mM at 2 mM intervals to 24 mM $MgC1_2$. Folding was carried out by rapid heat denaturation to 65°C followed by slow cooling from 65 to 60°C over 50 min, then 60 to 24°C over 72 h. We performed electrophoresis on samples using 2% Agarose gel (0.5X Tris/Borate/EDTA (TBE), 11 mM $MgC1_2$, 10 µL NA-red) at 70 V for 3.5 h in an ice-water bath. Discrete bands were visualized with UV trans-illuminator (Peiqing JS-680B). The desired bands were physically excised, crunched and filtered through a Freeze 'N' Squeeze spin column at 4°C for 10 min at 16000 ×g.

TEM imaging

Transmission electron micrographs were obtained with a HITACHI H-7650B TEM (Hitachi, Japan). A 3 µL DNA origami sample solution was deposited onto the carbon-coated side of the TEM grid and allowed to adsorb for about 5 min. The sample-side of the grid was then immersed in a 2% uranyl acetate stain-solution droplet and incubated for 40 s. Excess liquid was dabbed off with the edge of a filter paper, and the grid allowed to dry completely. Images were taken at 80.0 kV accelerating voltage.

RESULTS AND DISCUSSION

The square lattice based caDNAno software was used to design a 3D DNA origami cross-like structure (since the structure resembled a cross). Our design consisted of an overall 72-helix bundle which was all used to form the structure. The design consists of six layers having a total height of 12 nm. The width of the design consists of 12 helices thus the design is 24 nm wide. We estimated the length of the design from caDNAno to be approximately 38 nm. Therefore, the cross-like origami structure has approximate dimensions of 12 × 24 × 38 nm. Figure 1a shows a model depiction of the cross-like structure as designed. Figure 1b shows the folding scheme of the DNA origami cross-like structure. To prevent base stacking of the structures, we removed all the end sequences. We prepared a magnesium screen covering seven different concentrations from 12 mM at 2 mM intervals to 24 mM $MgCl_2$. The quality of folding was assessed by running a 2% agarose gel electrophoresis of the folded structures. The origami solutions containing the respective $MgCl_2$ salts are shown in Figure 2a. Lanes

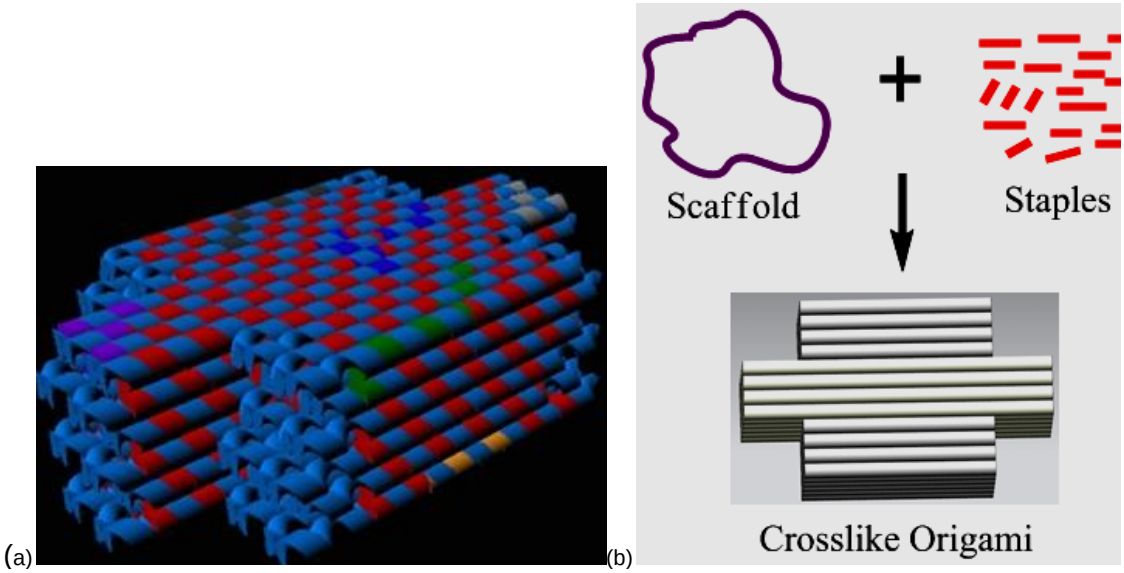

Figure 1. a. Model depiction of the 3D DNA origami cross-like structure. The scaffold strand is shown in light blue color, while the other colors represent the staple strands. **b.** Folding scheme of the DNA origami cross-like structure.

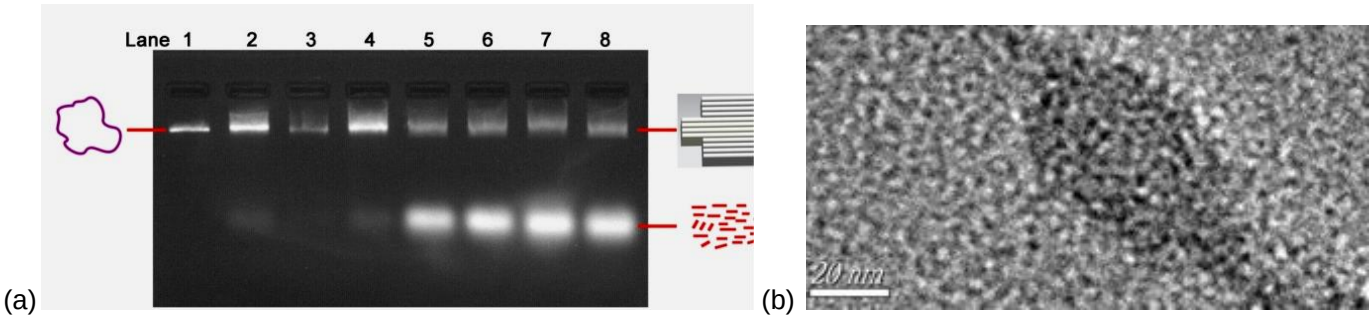

Figure 2. 2% agarose gel electrophoresis of the samples in the magnesium screen. i) Lane 1 contains M13mp18, ii) lanes 2 to 8 contained, respectively solutions from 12 to 24 mM Magnesium salts. The leading bands of lanes 2 to 8 were excised and purified for our objects and under the bands are excess staples. (b) TEM image of the 3D DNA origami cross-like structure with the scale bar indicated. Scale 20 nm.

2 to 8 in Figure 2a contained respectively 12 mM^{-24} mM $MgCl_2$. The band that contained the 12 mM salts was the fastest migrating and also the clearest. We physically excised this band and purified the structures via centrifugation using freeze 'n' squeeze DNA gel extraction spin columns. We performed transmission electron microscopy (TEM) on the purified structures and realized that the structures folded in 12 mM $MgCl_2$ yielded the best results. The TEM image of a single cross-like structure is shown in Figure 2b. In the figure, we see the clearly formed cross-like structure. Since the size of the single structure was very small, it was difficult imaging them. The length and width of the structure were determined from the TEM to be ~36 and ~25 nm. The length and width were caDNAno designed to be ~38 and 24 nm, respectively. The slight deviations could be attributed to the preparations on the TEM grid.

The use of biotin-streptavidin as a linking method has a long history. Biotin is a water soluble B-vitamin and is present in all living things in minute amounts. Biotin is a very small molecule and when used in biotinylation, does not usually alter many properties of the structures (Diamandis and Christopoulos, 1991). Streptavidin is a 52 kDa protein found in *Streptomyces avidinii*. Streptavidin is a symmetric tetramer which forms a brick with dimensions of 6 × 5 × 4 nm and a pair of biotin binding sites per each of the two 6 × 4 nm faces (Ringler and Schulz, 2003). It does have four high affinity binding sites for biotin and the binding of biotin to streptavidin is one of the strongest non-covalent interactions known

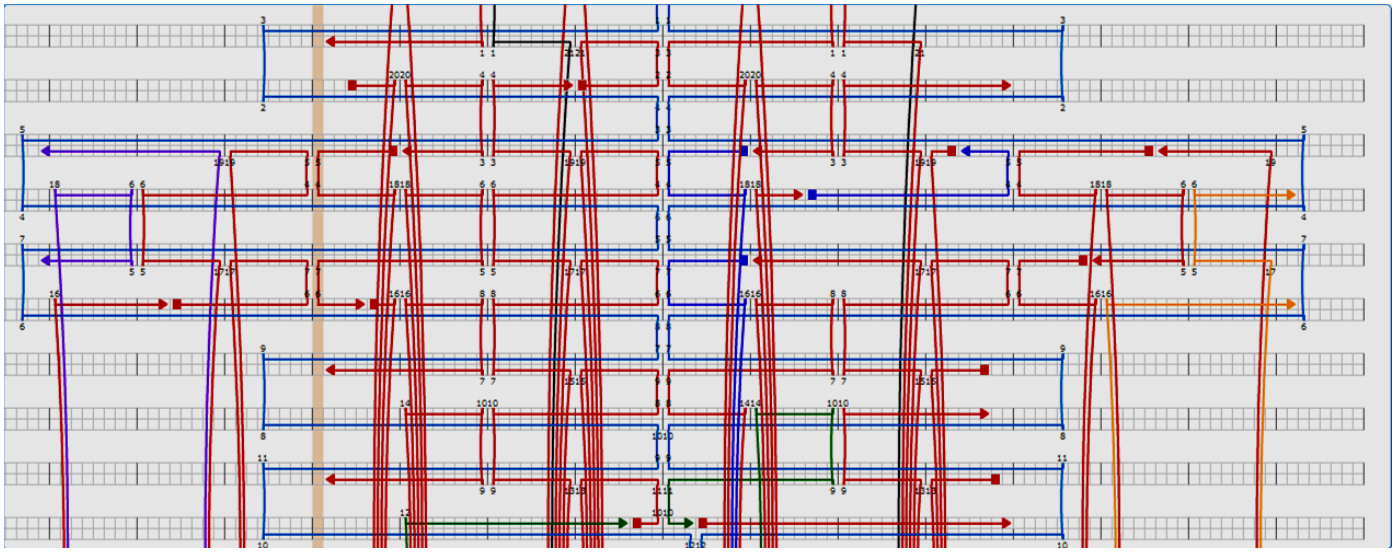

Figure 3. caDNAno interface of a section of the Path panel showing two designed connection sites (shown with violet and yellow colors).

in nature. The high affinity constant of interaction of biotin with streptavidin is about 10 times greater than the interaction of ligands with specific antibodies which ensures that the complex is stable even in harsh conditions of pH changes and multiple washings. The binding of biotin to streptavidin is very specific such that the binding is directed only to the target. One of the participating components in the biotin-streptavidin system should always be biotinylated (Diamandis and Christopoulos, 1991). Many scientists have utilized this biotin-streptavidin high affinity interaction to direct several processes and applications (Chiruvolu et al., 1994; Eskelinen et al., 2011; Lyonnais et al., 2008; Qi et al., 2005; Ringler and Schulz, 2003). Many of the research associating DNA origami with the biotin-streptavidin interaction has focused on decorating the origami template with other functional materials (Eskelinen et al., 2011; Lyonnais et al., 2008).

In order to connect the DNA origami cross-like structures using biotin-streptavidin interaction, we designed the sites where the connections were to be made. These connection sites were designed at the two extreme ends of the long axis of each of the cross-like structure. We designed two DNA single strands at each connection site. These two single strands were biotinylated at the ends (3' ends) that extend outside the origami structure as shown in figure 3. The connection sites are shown in Figure 3 indicated with violet and yellow colors. The two designed DNA single strands at each of the connection sites were extended with an 8 base sequence ATGCATGC for sufficient flexibility to the DNA strands so that the structure will not be strained. This is significant since it makes it easier for biotinylated strands from two structures to have easy access to the binding streptavidin. To connect the cross-like structure to another cross-like structure at the two positions indicated with violet and yellow colors in figure 3, all the generated sequences from caDNAno were used in addition to the sequences from the two designed connection sites that are extended with biotin.

Figure 4 shows the connecting process of three DNA origami cross-like structures using biotin-streptavidin interaction. After annealing and purification of the folded cross-like structures, we added a 20 µM 20 µl solution of streptavidin to a purified 20 µl solution of the origami cross-like structures and the mixture was incubated overnight at 4°C. After incubation, unbound streptavidin was removed using spin column filtration. The resulting solution was again washed with EDTA. After washing, the solution was mixed with purified 20 µl of the origami solution and the mixture incubated for two days at 4°C. Since streptavidin has four binding sites to biotin, one streptavidin molecule will bind two cross-like structures together. When streptavidin was added to the annealed DNA origami solution, two of its binding sites bonded with the two biotin molecules attached to the DNA origami template at one side. The same process also happens at the opposite side of the DNA origami template. In all the situations, two binding sites on the streptavidin are left free to bind to the biotin attached to the new DNA origami structure. We characterized the structures with TEM.

Figure 5 shows the TEM images of the connected structures at three different resolutions. We see in the images (Figure 5a to 5c) that the structures are only connected at the sites (ends of the long axis) where we have biotinylation. This shows that the connection came about through the biotin-streptavidin interaction and not base stacking interactions since we removed all the end sequences that are responsible for base stacking interactions. Since we biotinylated both lateral ends of the

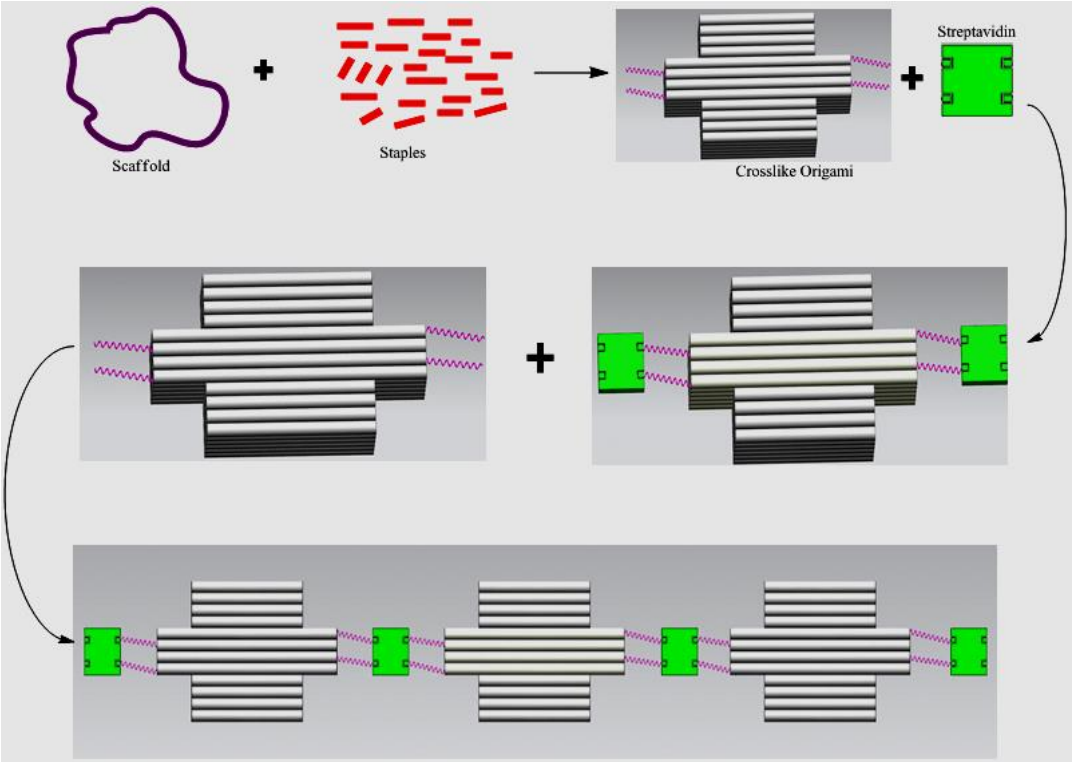

Figure 4. Design schematic of connecting three DNA origami cross-like structures together using the biotin-streptavidin interaction. As a first step, DNA origami cross-like structures (folding of scaffold by staple strands) biotinylated on two opposite sides with four different DNA strands are mixed with streptavidin to form the origami-streptavidin complex. The origami-streptavidin complex is purified to remove excess streptavidin and mixed with biotinylated origami structures to form the connected origami structures.

cross-like structure, it is possible for the connections to continue until both biotinylated strands and attached streptavidins are used up (Figures 5). Figure 5a shows only two DNA origami cross-like structures connected with a total length of approximately 69 nm. Figures 5b and 5c show the connection of five and four origami structures, respectively. The approximate lengths determined from the TEM images are respectively 207 nm for the five cross-like structures in Figure 5b and 145.5 nm for the four structures in Figure 5c. These lengths compare favorably with the length determined from caDNAno which has already been stated. We also observed that longer chains were not formed as we had assumed from the design. This could be that the streptavidin used was not enough. The fact that longer chains were not formed may also be due to the incubation period which might be short.

Conclusion

DNA origami structures can bind end-to-end through sticky end interactions. These interactions are always undesirable and efforts are made to remove them. Even if these interactions are needed, they are not strong enough. We have demonstrated the use of the biotin-streptavidin interaction to connect DNA origami structures even though longer chains were not realized. This interaction is very strong and could be used to form DNA origami networks. Assembly of two different configurations has been shown. These configurations consist, respectively, of two, four, and different cross-like origami structures which are connected together end-to-end. It was difficult quantifying the overall yields of our structures, but it was easy to find many structures on the TEM grids sufficient for our characterization analyses.

Conflict of interests

The authors did not declare any conflict of interest.

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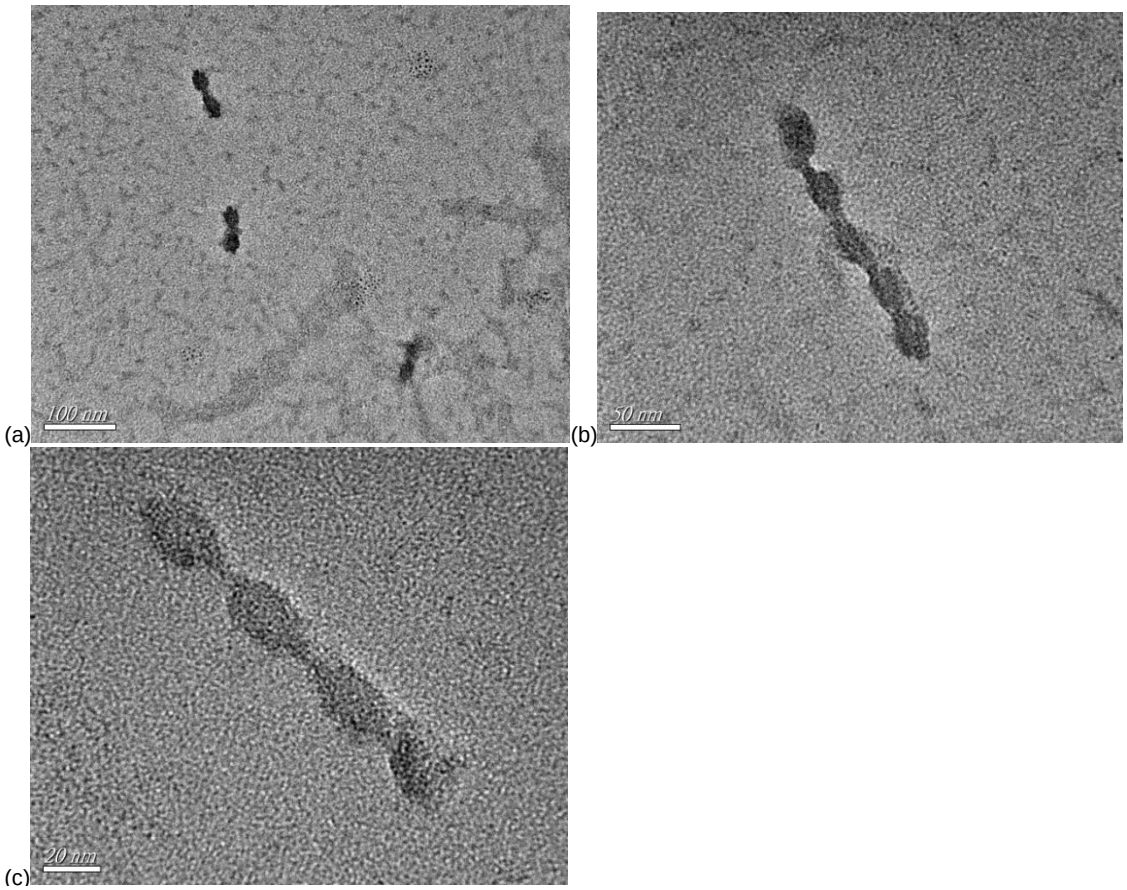

Figure 5. TEM images of the connected origami structures at two opposing connecting sites. Different scales (a) 100 nm, (b) 50 nm, and (c) 20 nm showing the lateral connections of the structures.

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Full Length Research Paper

Reproductive performance of dairy cows affected by endometritis, pododermatitis and mastitis

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The effects of endometritis, pododermatitis and clinical mastitis on the conception rate and calving-conception interval of multiparous and primiparous cows after fixed-time artificial insemination (FTAI) were evaluated. Clinical endometritis was diagnosed by ultrasonography 20-40 days postpartum upon observation of fluid in the uterine lumen. Cows with clinical endometritis were treated intramuscularly with 2 mg/kg ceftiofur hydrochloride over three consecutive days. Forty-five days after delivery, multiparous and primiparous cows with normal uteri according to ultrasonography were selected for the study, filed and inseminated by FTAI. To identify animals with hoof problems and clinical mastitis and to define their respective groups, the cows were observed daily during morning and nightly milking for up to 60 days after FTAI, and animals with hoof lesions were treated. Animals with clinical mastitis were treated with intramammary infusion containing 88 mg cefquinome sulphate every 12 h after milking for four days. The conception rate of multiparous cows with clinical endometritis at 30 and 60 days after FTAI was negatively affected compared with that of healthy cows with pododermatitis. However, clinical endometritis did not influence the primiparous category, whereas pododermatitis and clinical mastitis did not influence the conception rate of any category at 30 and 60 days after FTAI. Differences were not observed between primiparous or multiparous cows in the calving-conception interval.

Key words: Lactation, pregnancy, health, fertility.

INTRODUCTION

The postpartum period is critical for the remainder of a cow's reproductive life (Dohmen et al., 2000). Uterine infections correspond to an increase in the calving interval; discard rate and services required per conception and to a reduction in production (Leblanc et al., 2002; Sheldon et al., 2008). Infections of cattle limbs

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cause stress and reduce fertility and pregnancy rates (Sheldon, 1997). Cows with lameness have a higher number of services per conception, longer period of service and increased incidence of metritis and mastitis than healthy cows (Souza et al., 2006). Another disease that can influence the reproductive performance of cows is mastitis, which is a major concern in dairy cattle production (Carneiro et al., 2004). The mechanisms by which mastitis interferes with embryonic survival and mortality are not fully understood, but studies have shown that there may be a relationship between mastitis and reduced pregnancy rates (Hansen et al., 2004).

This study therefore aimed at evaluating the effects of clinical endometritis, pododermatitis and clinical mastitis on the conception rate and calving-conception interval of primiparous and multiparous lactating dairy cows after fixed-time artificial insemination (FTAI).

MATERIALS AND METHODS

The experiment was conducted on a dairy farm located in the municipality of Montividiu, southwest Goiás State, Brazil (latitude 17°20'5.7" and longitude 51°18'46.7"). During the experiment, lactating Holstein cows were confined in wooded feedlots supplied with drinking troughs. The cows received a complete diet consisting of quality corn silage and a balanced concentrate for milk production, which was distributed four times with the aid of a mixing wagon. The effects of endometritis, pododermatitis and clinical mastitis on the conception rate and calving-conception interval of multiparous and primiparous cows after FTAI were evaluated from May to August, 2013. The experimental animals (n = 356) were divided into two categories: multiparous cows, with an average milk production of 26.7± 3.6 kg milk/day, and primiparous cows, with an average milk production of 20.4 ± 2.8 Kg milk/day. Between 20 and 40 days after birth, the animals were reproductively evaluated by ultrasonography. Cows with clinical endometritis determined by observations of fluid in the uterine lumen were treated with 2 mg/kg ceftiofur hydrochloride (CEF50®, Agenor União Saúde Animal, Embu-Guaçu, SP, Brasil) intramuscularly for three consecutive days.

Animals with uteri without signs of infection upon clinical examination and ultrasonography (Mindray® DP3300 VET) were selected for the study after the voluntary waiting period of 50 days. The age of the experimental animals was 62.3 ± 7.4 days on average after birth, and they had a body condition score between 2.5 and 3.5 on a scale from 1 to 5, with 1 indicating very thin and 5 indicating very fat (Ferguson et al., 1994). The cows were synchronised and inseminated at a fixed time according to the following protocol: on the first day (D0), the cows received an intravaginal progesterone implant (Cronipres®, Biogénesis-Bagó, Garin, Buenos Aires, Argentina) and intramuscular application of 2 mg oestradiol benzoate (Bioestrogen®, Biogénesis-Bagó) After eight days (D8), the implant was removed, and 0.15 mg sodium cloprostenol (Croniben®, Biogénesis-Bagó), 300 UI equine chorionic gonadotropin (Folligon®, Intervet International B.V., Boxmeer, Holland) and 1 mg oestradiol cypionate (ECP®, Pfizer, Pharmacia and Upjohn Company, NY, USA) were administered. Forty-eight hours after implant removal (D10), 0.004 mg buserelin acetate Cows were observed daily during morning and afternoon milking for up to 60 days after FTAI to identify animals with hoof problems and clinical mastitis. Animals with hoof lesions were restrained in a hoof trimming chute for cleaning and corrective treatment. Animals with clinical mastitis were medicated with an intramammary infusion containing 88 mg cefquinome sulphate (Cobactan VL®, Intervet International B.V.) after milking and every 12 h for four days. (Sincroforte®, Ouro Fino, Cravinhos, SP, Brasil) was administered intramuscularly, and the artificial insemination was performed.

Thus, the groups were divided into healthy cows (n = 106), cows treated for clinical endometritis (n = 83), cows affected by pododermatitis (n = 97) and cows suffering from clinical mastitis (n = 70), within each category of multiparous and primiparous. Pregnancy was diagnosed at 30 and 60 days after FTAI through ultrasonography with a 5.0 MHz linear transducer (DP 3300, Mindray, Nanshan, Shenzhen, China).

Data were statistically analysed using R software (R Core Team, 2014). Comparisons of the conception rate were performed by the nonparametric chi-square test, whereas the average calving-conception interval was analysed by Tukey's test, both with a 5% significance level.

RESULTS

The conception rate at 30 days after FTAI of multiparous cows affected by and treated for clinical endometritis (18.8%) was significantly lower than the conception rate of healthy cows (41.6%, P = 0.0262) and cows affected by pododermatitis (41.7%, P = 0.0170). However, multiparous cows affected by and treated for clinical endometritis had the same reproductive performance as cows affected by clinical mastitis (35.3%). For primiparous cows, neither of the evaluated diseases affected the conception rate at 30 days after FTAI compared with healthy cows (Table 1). For the diseases evaluated in this study, only clinical endometritis (15.6%) affected the conception rate at 60 days after the FTAI of multiparous cows compared with that of healthy cows (37.3%, P = 0.0389). Differences were not observed between the other groups of multiparous cows or primiparous cows (Table 2). Table 3 shows that in both categories of cows (multiparous and primiparous); differences were not observed in the calving-conception interval of healthy cows and cows affected by the diseases evaluated here.

DISCUSSION

In the multiparous category, the conception rate at 30 and 60 days after the FTAI of cows affected by endometritis was significantly lower than the conception rate of healthy cows. These results are consistent with a study conducted nearly three decades ago in which more cows with normal puerperium became pregnant in the first service (42%) compared with cows with some postpartum abnormality (24%) (Benmrad and Stevenson, 1986). Similar results were not observed in the primiparous cows of the study. Studies have found that the presence of endometritis has no effect on fertility when considering the conception rate at first insemination (Kasimanickam et al., 2006). The same authors explained that the use of hormonal protocols for FTAI, such as ovsynch or presynch, promotes uterine immune

Table 1. Conception rates at 30 days after the fixed-time artificial insemination of multiparous and primiparous health dairy cows, cows treated for clinical endometritis, cows affected by pododermatitis and cows suffering from clinical mastitis.

Category	Conception rate n (%)			
	Healthy	Endometritis	Pododermatitis	Mastitis
Multiparous (n = 205)	41.6[a] (26/62)	18.8[b] (9/47)	41.7[a] (24/58)	35.3[ab] (13/38)
Primiparous (n = 151)	43.8[a] (19/44)	28.6[a] (10/36)	33.3[a] (13/39)	22.2[a] (7/32)

Values followed by different letters in the same row are significantly different (P < 0.05).

Table 2. Conception rate at 60 days after the fixed-time artificial insemination of multiparous and primiparous health dairy cows, cows treated for clinical endometritis, cows affected by pododermatitis and cows suffering from clinical mastitis.

Category	Conception rate n (%)			
	Healthy	Endometritis	Pododermatitis	Mastitis
Multiparous (n = 205)	37.3[a] (23/62)	15.6[b] (7/47)	33.3[ab] (19/58)	29.4[ab] (11/38)
Primiparous (n = 151)	41.3[a] (18/44)	28.6[a] (10/36)	33.3[a] (13/39)	22.2[a] (13/39)

Values followed by different letters in the same row are significantly different (P < 0.05).

Table 3. Mean (± SD) of the calving-conception interval (in days) of multiparous and primiparous healthy dairy cows, cows treated for clinical endometritis, cows affected by pododermatitis and cows suffering from clinical mastitis.

Category	Average calving-conception interval ± Standard Deviation (days)			
	Healthy	Endometritis	Pododermatitis	Mastitis
Multiparous	149.94 ± 68.46 a	169.37 ± 96.02 a	188.80 ± 104.11 a	174.13 ± 116.46 a
Primiparous	156.69 ± 68.61 a	162.33 ± 111.79 a	181.00 ± 80.01 a	175.62 ± 86.42 a

Averages followed by the same letter on the same row do not differ from each other (P < 0.05).

mechanisms, thereby minimising the effects of endometritis. However, several studies have shown that uterine infections caused economic losses to livestock because they increase the number of services and reduce production (Andrade et al., 2005).

Uterine discharge does not affect the number of services per conception, although the calving interval and first insemination is longer in animals with endometritis (Williams et al., 2005). Differences in the calving-conception interval between healthy cows and cows treated for endometritis in multiparous (149.94 ± 68.46 versus 169.37 ± 96.02) and primiparous (156.69 ± 68.61 versus 162.33 ± 111.79) cows were not observed in this study. These results are consistent with the results of another study in which the calving-conception interval did not change in cows that had endometritis, were treated by intra-uterine infusions of ceftiofur and received PGF2α in the oestrus synchronisation protocol (P >0.05) (Galvão et al., 2009).

Recent studies have shown that cows treated for endometritis experienced an average delay of 28 days in the calving-conception interval, and this delay can cause greater economic losses than what is experienced during disease treatment because a reduction in the conception rate and increase in the calving-conception interval cause a longer calving interval and lower milk production (Marques et al., 2012). These data are consistent with another study that reported a longer calving-conception interval for cows treated for endometritis compared with healthy cows (151 versus 119 days) (Leblanc et al., 2002).

Compared with the results obtained in this study, Melendez et al. (2003) showed that lame cows had lower conception rates than the control group at first service (17.5% versus 42.6%). Studies have shown that hoof problems reduced the ovarian activity of Holstein cows at 60 days postpartum (Gabarino et al., 2004), which is important because cows must experience ovarian cycling

to expel contaminants from the uterus (Benmrad and Stevenson, 1986). Another study observed conception rates at first service of 56 and 46% in cows without hoof problems and cows with lameness, respectively (Collick et al., 1989). These rates are greater than the values observed in this study, and this difference can be explained by the fact that oestrus synchronisation was performed in Collick et al. (1989) and FTAI was performed in the study.

In this study, the calving-conception interval was not affected by hoof lesions in the multiparous (149.94 ± 68.46 versus 188.80 ± 104.11) and primiparous (156.69 ± 68.61 versus 181.00 ± 80.01) cows. However, the multiparous cows with pododermatitis tended (P = 0.0937) to have a longer calving-conception interval, which was 38.86 days greater than that of the healthy cows. When the hoof lesions affected the hindlimbs and forelimbs, the calving interval at first service increased by 2.9 and 4.6 days, respectively (Barkema et al., 1994). Other authors have reported that lame cows showed longer service periods than healthy cows (266 versus 200.5 days, respectively) and an increased incidence of metritis (25% versus 12.5%) and mastitis (60% versus 29%) compared with normal cows (Souza et al., 2006). Lame cows feel pain and stress that may predispose them to certain diseases, such as metritis and mastitis, and increased glucocorticoids, which cause premature luteolysis (Melendez et al., 2003).

In this study, the primiparous cows affected by mastitis (22.2%) tended to have a lower conception rate than healthy cows (43.8%) at 30 days (P = 0.0608). However, the embryonic and foetal mortality between 30 and 60 days was low for multiparous cows and absent in primiparous cows suffering from mastitis. A reduced conception rate in animals with mastitis may be related to the mechanism of maternal recognition because mastitis promotes the production of several bioactive molecules that can disrupt the functioning of the reproductive system (Slebodzinski et al., 2002).

According to the literature, prostaglandins are under the control of a several cytokines, including tumour necrosis factor-α (TNF-α) and interleukin (IL)-1α, which can increase the secretion of prostaglandin F2α (PGF2α) by the endometrium (Skarzynski et al., 2000). It has been shown that there is an increase of mRNA for IL-1α, IL-1β, TNF-α, IL-10 and IL-12 and protein for TNF-α in cells derived from milk that cause an infection of mammary-glands. Thus, the release of cytokines into blood during mastitis can induce the release of PGF2α and premature luteolysis (Waller et al., 2003).

Premature luteolysis was observed in cows suffering from mastitis, although it was not observed in normal cows. Furthermore, the oestrus cycle was longer when mastitis occurred during the follicular phase, and these results indicate that mastitis can affect postpartum ovarian activity in dairy cows (Huszenicza et al., 2005). Differences were not observed in the calving-conception interval of cows that were healthy and affected by mastitis in both the multiparous (149.94 ± 68.46 versus 174.13 ± 116.46, respectively) and primiparous categories (156.69 ± 68.61 versus 175.62 ± 86.42, respectively).

Although significant differences were not observed in the calving-conception interval of the categories evaluated in the study, dairy farmers can suffer economic losses because of disease treatment and lower milk production. Therefore, adequate nutritional, sanitary and reproductive management can help reduce unnecessary expenses that can be converted into revenue.

Conclusion

Based on the results obtained in this study, endometritis was shown to affect the conception rate of multiparous cows at 30 and 60 days after FTAI, although it does not influence the primiparous cows. Pododermatitis and clinical mastitis do not affect the conception rate of either the multiparous or primiparous cows at 30 and 60 days after FTAI.

Conflict of interests

The author(s) did not declare any conflict of interest.

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Ethics committee

The study was approved by the Ethics Committee of the Goias Federal Institute of Education, Science and Technology (Instituto Federal de Educação, Ciência e Tecnologia Goiano – IF Goiano) and filed under protocol number 033/2012.

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