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ANTIMICROBIAL ACTIVITY OF CALLUS AND CELL SUSPENSION CULTURES EXTRACTS OF *Thevetia peruviana*[a]

ACTIVIDAD ANTIMICROBIANA DE CULTIVOS DE CALLOS Y SUSPENSIONES CELULARES DE *Thevetia peruviana*

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ABSTRACT: *Thevetia peruviana* is an ornamental plant considered as a source of biological compounds with cardiac and antimicrobial activity. These compounds are normally extracted from different parts of *in vivo* plants. In this work, extracts were obtained from *in vitro* callus and plant cell suspension cultures of *T. peruviana* and their antimicrobial activity was evaluated by disk diffusion tests against gram negative (*Salmonella thipimurium* and *Escherichia coli*) and gram positive (*Staphylococcus aureus* and *Bacillus cereus*) strains. Ethanol, methanol and hexane extracts from callus and cell suspension cultures showed biological activity. Methanolic cell suspension extract showed activity against *B. cereus* and *S. aureus*. Ethanolic cell suspension extract inhibit all the bacteria, especially *S. thipimurium* while hexanic extract showed resistance activity against *S. thipimurium*, *S. aureus* and *B. cereus*. In terms of the source of the extracts, hexane extracts obtained from cell suspension cultures showed a higher antimicrobial activity than callus, while ethanol extracts had an inverse behavior. These results outline *in vitro* cell culture of *T. peruviana* as a feasible biotechnological platform for the production of compounds with antimicrobial activity..

KEYWORDS: Antimicrobial activity; callus culture; plant cell suspension culture; *Thevetia peruviana*.

RESUMEN: *Thevetia peruviana* es una planta ornamental considerada fuente de compuestos biológicos con actividad cardiotónica y antimicrobiana extraídos normalmente de diferentes partes de la planta. En este trabajo se obtuvieron extractos a partir de cultivos de callos y suspensiones celulares de *T. peruviana* y se evaluó su actividad antimicrobiana mediante pruebas de difusión en disco contra cepas gram negativas (*Salmonella thipimurium* y *Escherichia coli*) y gram positivas (*Staphylococcus aureus* y *Bacillus cereus*). Los extractos de etanol, metanol y hexano de cultivos de callos y suspensiones celulares mostraron actividad biológica. El extracto de metanol de la suspensión celular mostró

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actividad contra *B. cereus* y *S. aureus*. El extracto etanólico de suspensión celular inhibió todas las bacterias, especialmente *S. thipimurium*, mientras que el extracto hexanico mostró actividad de resistencia contra *S. thipimurium*, *S. aureus* y *B. cereus*. En cuanto a la fuente de los extractos, los extractos de hexano obtenidos de los cultivos en suspensión celular mostraron una mayor actividad antimicrobiana en comparación con el callo, mientras que los extractos de etanol tuvieron un comportamiento inverso. Estos resultados perfilan el cultivo celular *in vitro* de *T. peruviana* como una plataforma biotecnológica factible para la producción de compuestos con actividad antimicrobiana.

PALABRAS CLAVE: Actividad antimicrobiana; cultivo de callos; cultivo de células en suspensión; *Thevetia peruviana*.

1. INTRODUCTION

Plants are known for their ability to synthesize a variety of compounds, which have proved to be useful for the treatment of several diseases. It is estimated that approximately two thirds of the active ingredients of anticancer agents and drugs against infectious diseases are derived from plants (McChesney *et al.*, 2007).

Wide variety of metabolites produced by plants are employed by food, chemical, pharmaceutical and cosmetics industries for the production of multiple products and medicines, in a growing market of billions of dollars a year (Sasson *et al.*, 1992).

Within the pharmaceutical industry, antibiotics are the first choice for the treatment of infections. However, continuous and unsupervised uses of currently available antibiotics by the healthcare and agricultural systems have resulted in several bacterial strains (i.e. *Bacillus subtilis*, *Bacillus cereus* and *Escherichia coli*) resistant to these antibiotics. These scenarios make it difficult to implement effective treatments against bacterial infections. In order to overcome this problem, plant extracts with antimicrobial activity, have been used as a complement in order to enhance the activity against resistant bacteria (Hammer *et al.*, 1999; Rahman *et al.*, 2014). In addition, in recent years, the use of plant extracts with antimicrobial activity which improve the effectiveness of topical creams and lotions has increased significantly (Kareru *et al.*, 2010a).

Thevetia peruviana (*T. peruviana*) is a commonly ornamental shrub that belongs to the Apocynaceae family. It is considered a potential source of biological active compounds, exhibiting anti-termite (Kareru *et al.*, 2010b; Tagbor, 2009), antimicrobial (Hassan *et al.*, 2011; Kareru *et al.*, 2010b; Kareru *et al.*, 2010a; Rahman *et al.*, 2014) anti HIV (Tewtrakul *et al.*, 2002) and anticancer (Newman *et al.*, 2008) activities, among others. *T. peruviana* is particulary know for the production of cardiac glycosides such as thevetin, neriifolin and peruvoside, used as alternative drugs to digoxin in the treatment of heart failure (Zibbu & Batra, 2011).

In the last 15 years, the antimicrobial potential of *T. peruviana* extracts (leaves, fruits and roots explants) has been intensively studied (Bhoyar & Biradar, 2014; Gezahegn *et al.*, 2015; Reddy, 2010). Reports by several authors have shown promising antimicrobial activity for several extracts, standing out those with presence

of phenolic compounds and flavonoids (Cushnie & Lamb, 2005). However, not unanimity as to the solvents used, sensitive bacteria and inhibition halos has arisen from these studies.

Establishment of *in vitro* cell cultures of *T. peruviana* could provide a new biotechnological platform for the production of high value compounds with commercial interest (Arias *et al.*, 2010), including antimicrobial agents. Few studies have evaluated the antimicrobial activity of *in vitro* cell cultures of *T. peruviana* (Alhashimi *et al.*, 2013). However, to the best of our knowledge, there has not been published any work which compare and correlated callus and cell suspension cultures with the antimicrobial activity. Therefore, this work aims to evaluate the antimicrobial potential of different extracts from callus and cell suspension cultured of *T. peruviana*.

2. MATERIALS AND METHODS

2.1. Plant material, callus and cell suspension cultures

Callus and cell suspension cultures were obtained following the protocol described by (Arias *et al.*, 2010). Fruits were disinfected through washes with ethanol (70 % v/v), sodium hypochlorite (10 % w/v) and sterile distilled water. Aseptic pulp explants were sowed in solid Schenk and Hildebrandt (SH) medium supplemented with sucrose (30 g/L), 2.4-D (2 mg/1), kinetin (0.5 mg/L) and agar-agar (7 g/L). Explants were subcultured every 15 days in fresh medium until friable callus were obtained.

Cell suspension cultures were obtained through transfer of 2 g of friable callus to 100 mL of liquid SH medium in a 250 mL flask. Cell suspensions were maintained under constant agitation (110 rpm), 25$\pm$3°C and natural photoperiod (12h day light/12h darkness). Suspension cell cultures were subcultured every 12-15 days.

Fresh biomass obtained from friable callus and cell suspensions were freezed/dried (SYCLON-18N) at -60°C and 1 bar during 48 hours to avoid degradation of thermolabile compounds.

2.2. Phytochemical analysis

Preliminary phytochemical analysis was conducted according to some modifications to the standard protocol described by Harborne (1873). 2 g of freezed/dried biomass from callus and cell suspension cultures were extracted with 50 mL of solvent, then each extract was concentrated to a volume of 2 mL in a rotary evaporator (IKA HB10 control) at 40°C, 110 rpm and 145 psi. Each extract was used according to the methodology described for each family of metabolites to be identified according to Harborne (1973) for Thin Layer Chromatography (TLC) determination. Compounds families and tests performed are shown in Table 1. Results are reported on a qualitative scale as negative or positive: low (+), medium (++), or high

(+++).

Table 1: Metabolites to identify using Thin Layer Chromatography (TLC).

Metabolite	Test
Alkaloids	Dragendorff
	Mayer
	Valser
Steroids	Lieberman-Bourchard
Flavonoids	Shinoda
Phenols and tannins	FeC_{l3} and Jelly
Saponins	Foam
Anthroquinones	Brötager
Sesquiterpenic lactones	Baljet
	Kedde
	Legal
Cardiac glycosides	Kedde
	Legal

2.3. Callus and cell suspension culture extracts preparation

In order to make an exploratory study of various crude extracts eight solvents of different polarities were used for extractions; in ascending polarity (methanol, ethanol, acetone, ethyl acetate, dichloromethane, chloroform, hexane and petroleum ether). About 1.5 g of freezed/dried biomass (callus and cell suspensions) was used, for each extract, in a Soxhlet apparatus with 100 mL of solvent during five siphon cycles. Solvents were evaporate in their entirety under reduce pressure (145 psi) using a rotary evaporator system (IKA RV 10 control) at 40°C using a water bath (IKA HB 10 control). Each extract was diluted in 1 ml of dimethylsulfoxide (DMSO) 6 % v/v, obtaining the maximum extract concentration to be evaluated. All the extracts were stored at 4°C in the dark until use.

2.4. Bacterial strains and cultures

Tested bacterial included two gram-negative strains: *Salmonella thipimurium* (*S. thipimurium*) (ATCC 14028), *Escherichia coli* (*E. coli*) (ATCC 8739) and two gram-positive strains: *Staphylococcus aureus* (*S. aureus*) (ATCC 49775), *Bacillus cereus* (*B. cereus*) (ATCC 10987). The main characteristics of the bacteria strains are described in Table 2.

Each strain was activated on nutrient agar 24 hours before susceptibility testing. Bacterial solution was prepared by transferring a loop-full of three colonies of each bacteria into test tubes containing 5 mL of saline solution (0.8 % weight/volume of sodium chloride). The bacterial concentration were adjusted to 0.5 McFarland standard (absorbance 0.08-0.13 at 625 nm) (Sutton, 2011).

Table 2: Main characteristics of the bacterial strains used in susceptibility testing.

Bacteria	Gram reaction	Shape	Aerobic growth	Anaerobic growth	Endospore formation
S. thipimurium	-	Rods	+	-	-
E. coli	-	Rods	+	-	-
S. aureus	+	Cocci	+	+	-
B. cereus	+	Rods	+	-	+

2.5. Antibacterial activity assay

Antibacterial activity assay was tested by agar diffusion test (Bauer *et al.*, 1966). A sterile swab, impregnated with the prepared bacterial solution was used to surface inoculate a petri dish with Mueller Hilton agar in order to obtain a homogeneous bacteria growth. 20 μL of each extracts were deposited in 6 mm diameter sterile discs (MN 827 ATD) which were placed on the surface of each inoculated petri dish. Petri dishes were stored at 4°C for 2 hours to allow the diffusion in the agar of the extracts before bacterial growth. Next, petri dishes were incubated at 36°C, between 24 and 30 hours. All organic solvents and DMSO were evaluated as the negative control and tetracycline at a concentration of 2 mg/disc was used as positive control. All the assays were performed in duplicate. Inhibition values were reported as the average total diameter (diameter disc + inhibition).

3. RESULTS AND DISCUSSION

3.1. Phytochemical analysis

Phytochemical analysis of callus and plant cell suspension cultures obtained by TLC is shown in Table 3. Both *in vitro* culture systems showed the presence of cardiac glycosides and flavonoids. In addition, cell suspensions showed the presence of phenolic compounds.

These results are consistent with those reported by previous research. Presence of cardiac glycosides in extracts of different parts of the *in vivo* plant (Gezahegn *et al.*, 2015; Kohls *et al.*, 2012; Mateus, 2012) as well as *in vitro* callus and suspension cultures (Alhashimi *et al.*, 2013; Arias *et al.*, 2010) had been reported. Other authors have detected presence of more families of secondary metabolites such as alkaloids, terpenoids, saponins, flavonoids and phenols (Arias *et al.*, 2016; Bhoyar & Biradar, 2014; Gezahegn *et al.*, 2015).

Differences in types of metabolites with other authors and among themselves may lie in the source of the plant used to obtain the extracts as well as the different growing conditions of the same.

Table 3: Phytochemical analysis in callus and plant cell suspension cultures of *T. peruviana*.

Metabolite	Callus	Cell suspension
Alkaloids	Negative	Negative
Steroids	Negative	Negative
Flavonoids	Positive +	Positive +
Phenols and tannins	Negative	Positive +
Saponins	Negative	Negative
Anthraquinones	Negative	Negative
Sesquiterpenic lactones	Negative	Negative
Cardiac glycosides	Positive +	Positive ++

Low: + Medium: ++ High: +++

3.2. Antibacterial activity of cell suspension cultures

Table 4 shows the final concentration for each extracts; note that the concentration of the extract decreases with decrease of solvent polarity.

Table 4: Extract concentrations obtained from cell suspension cultures of *T. peruviana.*

Solvent	Extract Concentration (mg/ml)
Methanol	475.6
Ethanol	454.5
Acetone	122.9
Ethyl Acetate	55.2
Dichloromethane	56
Chloroform	47.7
Hexane	59.7
Petroleum Ether	39.8

Methanol, ethanol and hexane extracts showed the best antibacterial activity. Representative results for *B. cereus* and *S. aureus* are shown in Figure 1.

The total diameter of inhibition presented by the extracts in all bacterial strains is shown in Table 5.

Methanol extracts showed activity against *B. cereus* and *S. aureus* with inhibition diameters of 11 and 9 mm, respectively (Table 5). Ethanol extract showed greatest inhibition against *S. thipimurium* (inhibition diameter of 14 mm) while for the remaining strains the inhibition diameters were of 9 mm. Hexane extract showed a resistance diameter of 23 mm for *S. thipimurium* and inhibition diameters of 17 and 19.3 mm for

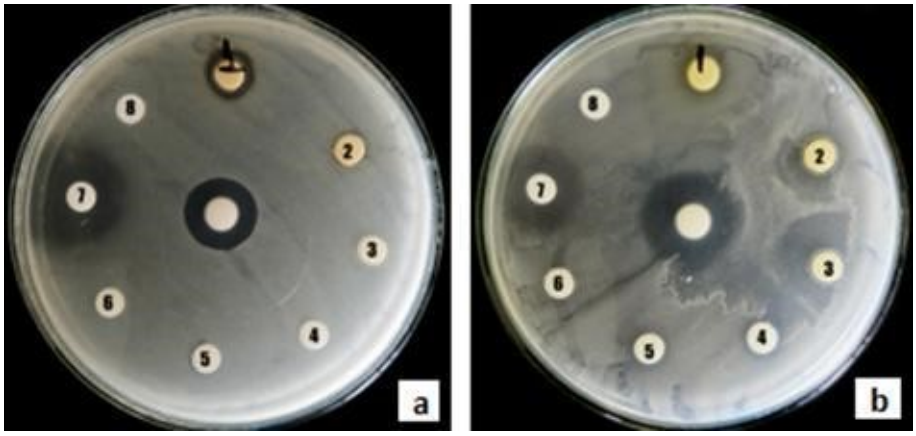

Figure 1: Antimicrobial activity of extracts obtained from cell suspension cultures of *T. peruviana*: *B. cereus* (a) y *S. aureus* (b). (1: methanol, 2: ethanol, 3: acetone, 4: ethyl acetate, 5: dichloromethane, 6: chloroform, 7: hexane, 8: petroleum ether, center: tetracycline). Source: Own elaboration.

S. aureus and *B. cereus*, respectively. In contrast, hexane did not show activity against *E. coli*. None of the others extracts showed antibacterial activity against more than one strain, as in the case of Ethyl Acetate who only presented an inhibition diameter of 11 mm against *S. aureus*.

Table 5: Total diameter of inhibition zone (mm) obtained with plant cell suspension culture extracts of *T. peruviana*. (M: Methanol, E: Ethanol, A: Acetone, EA: Ethyl Acetate, D: Dichloromethane, C: Chloroform, H: Hexane, PE: Petroleum Ether).

Solvent	Concentration (mg/disc)	Total diameter inhibition zone (mm)			
		E. coli	*S. thipimurium*	*B. cereus*	*S. aureus*
M	9.5	-	-	11	9
E	1.5	9.7	14	9	9
A	2.5	-	-	-	-
EA	1.1	-	-	-	11
D	1.1	-	-	-	-
C	1	-	-	-	-
H	1.2	-	23*	19.3	17
PE	0.8	-	-	-	-
Tetracycline	2	17.3	18	16	19

*: halo resistance

Gezahegn *et al.* (2015) observed increased antibacterial activity for ether extract from leaves of *T. peruviana* against *E. coli* (13.50 mm) and *S. typhimurium* (16.50 mm), while the acetone extract was more effective against *S. aureus* (17.00 mm). Naz and Agrawal, working with ethanol extracts of leaves of *T. peuruviana* observed a concentration depend antibacterial activity for *E. coli* and *S. aureus* (Naz & Agrawal, 2015). Rahman *et al.* (2014) reported for ethanol extracts of leaves and fruits (1.5 mg/well), maximum inhibition diameters for *E. coli* of 10.33 and 9.33 mm, respectively. Alhashimi *et al.* (2013), compared the activity of

alcohol extracts of leaves and callus of *T. peruviana*, seeing increased antibacterial activity in callus extracts against *S. aureus* (15 mm) and *P. aeruginosa* (13 mm) for the extracts of 40 mg/well and *B. cereus* (12 mm) to concentration of 35 mg/well. Both extracts showed resistance to *E. coli*. Moreover, Hassan *et al.* (2011), reported very low inhibition values for ethanol extracts of leaves at concentrations of 100 μg/disc on gram positive and gram negative bacteria, particularly *S. sonnei* (1.5 mm), *S. aureus* (2.7mm) and *B. cereus* (0 mm).

Other authors have reported antimicrobial activity of extracts obtained from leaves of *Thevetia neriifolia*, obtaining very good results against human pathogens (bacteria and fungi) for aqueous and hexane extracts (Buvaneswari *et al.*, 2011), ethanol, ethyl acetate, chloroform and methanol (Buvaneswari *et al.*, 2011; Nesy & Mathew, 2014).

Methanol, ethanol and hexane extracts showed a greater inhibitory effect on the inhibition growth of gram-positive bacteria than for gram-negative bacteria, which is in accordance with the reports described above.

The results obtained in this work for ethanol and hexane extracts are very similar to those reported by some of the previously mentioned authors. They have mainly worked with extracts obtained from fruits and leaves, indicating that the *in vitro* culture of cell suspensions of *T. peruviana* is a feasible strategy for the production of compounds with antimicrobial activity of alternative species. Because of this, it was evaluate the differential antibacterial activity that could occur in two different systems of *in vitro* plant cell cultures, callus and suspensions.

3.3. Effect of the *in vitro* culture (callus or suspensions) on the antibacterial activity of ethanol and hexane extracts

Callus and suspension hexane extracts (Table 6) did not show activity against *E. coli*, corroborating the results obtained in the sweep solvent made for cell suspensions (Table 5). Hexane extracts showed the same activity against *B. cereus* and *S. aureus*; in the case of *S. thipimurium* showed a higher antibacterial activity changing to a clear inhibition zone instead of a resistance halo; this change may be due to increased concentration tested, from 1.5 to 2 mg of extract per disc.

Suspension hexane extracts showed a slightly higher antibacterial activity than the obtained for callus against all tested bacteria strains. Moreover, callus ethanol extracts showed antibacterial activity slightly higher than that obtained for cell suspensions, except for *B. cereus*, which did not register any callus activity and very low activity for the suspensions (8 mm).
Callus antimicrobial activity observed in this study is higher compared to Alhashimi *et al.* (2013), who reached similar inhibition ranges but whit higher concentration of the extracts. The observed difference in activity may be due either to the source of explant used to obtain callus, Alhashimi *et al.* (2013) employed leaves instead of pulp fruit. In addition, the age of the culture used to obtain extracts as well as specific

Table 6: Total diameter of inhibition zone (mm) obtained by ethanol and hexane extracts of callus and cell suspensions of *T. peruviana*. (E: Ethanol, H: Hexane).

Plant material	Solvent	Concentration mg/disc	Total diameter of inhibition zone (mm)			
			E. coli	*S. thipimurium*	*B. cereus*	*S. aureus*
Suspension	E	1.6	9.7	14.0	8.0	13.3
Callus		1.4	14.7	16.7	-	15.0
Suspension	H	2.0	-	14.7	13.3	14.0
Callus		1.9	-	11.0	10.7	12.0
Antibiotic						
Tetracycline		2	15.7	16.0	16.3	16.3

endemic characteristics of the used plants can contribute to the observed differences.

Differences in antimicrobial activity between callus and cell suspensions extracts can be considered as evidence of the effect on the production of active compounds in two different culture systems (solid for callus and liquid for suspensions). This effect has been demonstrated in the case of *T. peruviana* by Alhashimi *et al.* (2013) who observed increased activity in alcoholic extracts obtained from *in vitro* cell callus compared to extracts of *in vivo* leaves.

Although a difference was observed in the antibacterial activity of the extracts obtained from callus and cell suspensions, it is quite complex to attribute this activity only to the difference observed in the phytochemical profile. TLC is a preliminary technique and does not allow to identify accurately neither the type of compounds nor the concentration of each of the families that were identified.

These results are encouraging to continue in the subsequent fragmentation of the extracts that showed to have biological activity. Identify the active compound, determine the IC50, purify it and eventually establish its biotechnological production on a larger scale.

4. CONCLUSIONS

The results of this study indicate that ethanol and hexane extracts obtained from *in vitro* cultures of *T. peruviana* (callus and suspensions) present antibacterial activity in the tested strains. Antibacterial activity was higher against gram-positive bacteria (*S. aureus* and *B. cereus*). *In vitro* cultures of *T. peruviana* showed similar antibacterial activity to those reported by other authors in previous works for *in vivo* plant extracts. These results establish this *in vitro* culture as a promising biotechnological platform for obtaining natural compounds with antimicrobial activity having the advantages of *in vitro* cultivation above *in vivo* cultures.

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