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Role of polyphenols in the antimicrobial activity of ethanol *Tamarindus indica* L leaves fluid extract

[Papel de los polifenoles en la actividad antimicrobiana del extracto fluido de las hojas de *Tamarindus indica L*]

Julio César ESCALONA-ARRANZ[1], Renato PÉREZ-ROSÉS[1,2], Imilci URDANETA-LAFFITA[1], Humberto MORRIS-QUEVEDO[3], María Isabel CAMACHO-POZO[3], Jesús RODRÍGUEZ-AMADO[1] & Gustavo SIERRA GONZÁLEZ[4]

[1]Pharmacy Department, Oriente University. Santiago de Cuba, Cuba
[2]Departament Farmacología i Química Terapéutica. Universitat de Barcelona, Spain
[3]Centre for Studies in Industrial Biotechnology. Oriente University, Santiago de Cuba, Cuba
[4]Finlay Institute. Ciudad de la Habana, Cuba
Contactos | Contacts: Julio César ESCALONA-ARRANZ - E-mail address: jcea@cnt.uo.edu.cu

Abstract
The aim of this work was to explore in an active, fractioned, and chemically characterized *Tamarindus indica* L. (TIL) leaves extract, the influence of flavonoids and polyphenol compounds on the antimicrobial activity. A spectrophotometric quantification of the total phenols and flavonoids content was determinate to the TIL leaves extract, as well as, to the four fractions in which was fractioned (n-hexane, chloroform, ethyl acetate and n-butanol). The extracts and their fractions were microbiologically tested against six ATCC bacteria and *Candida albicans*, being determined their minimum inhibitory and bactericidal concentrations (MIC and MBC). Additionally, the extracts were evaluated in their influence on human complement system (classical and alternative pathways). Fractions with high content of flavonoids and polyphenols (ethyl acetate and n-butanol) are active against *Bacillus subtilis* and inhibit the human complement system (direct pathway, IC_{50} 31.05 and 33.65 μg/mL respectively), but are not active over *Staphylococcus aureus*. However, this bacterium was susceptible to fractions with low or null concentration of flavonoid or polyphenol compounds. No fractions neither the fluid extract were active against *Salmonella typhimurium* and *Candida albicans.* Experimental data suggest that phenols and flavonoids are not the only components involved in the antimicrobial activity of TIL leaves as has been previously suggested by other authors. Complement activity tests did not support a putative role on the antimicrobial activity.

Keywords: tamarind, complement activity, flavonoids.

Resumen
El objetivo de este trabajo fue explorar en un extracto activo de hojas de *Tamarindus indica* L. (TIL), fraccionado y caracterizado químicamente, la influencia de los polifenoles y flavonoides en su actividad antimicrobiana. Se cuantificaron por espectroscopia UV-visible los contenidos de fenoles totales y flavonoides en el extracto de TIL así como de las cuatro fracciones obtenidas (n-hexano, cloroformo, acetato de etilo y n-butanol). Se evaluó la actividad microbiológica del extracto y sus fracciones contra seis bacterias ATCC y *Candida albicans*, determinándose sus concentraciones mínimas inhibitorias y bactericidas (MIC y MBC). Adicionalmente, se evaluó la influencia de los extractos en el sistema de complemento humano (vía clásica y alternativa). Las fracciones con altas concentraciones de polifenoles y flavonoides (acetato de etilo y n-butanol) fueron activas contra el *Bacillus subtilis* e inhibieron el sistema de complemento humano (vía directa, IC_{50} 31.05 y 33.65 g/mL, respectivamente), pero no fueron activas contra *Staphylococcus aureus*. Sin embargo, esta bacteria fue susceptible a fracciones con baja o nula concentración de polifenoles y flavonoides. El extracto fluido y todas sus fracciones resultaron inactivos frente a *Salmonella typhimurium* y *Candida albicans*. Los datos experimentales sugieren que los fenoles y flavonoides no son los únicos compuestos involucrados en la actividad antimicrobiana de hojas de TIL, como había sugerido por otros autores. La actividad medida sobre el sistema de complemento, no aporta relevancia a la actividad antimicrobiana de las hojas de TIL.

Palabras Clave: Palabras claves en español.

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Introduction

Tamarindus indica L. (*Caesalpiniaceae*) or tamarind as is usually known is a tropical tree used primarily for its fruits, which are eaten fresh or processed, used as a seasoning or spice, and as medicine beverages. Also, it has edible leaves, which are rich in fat, fiber, vitamins, proteins, flavonoids (Razali *et al*., 2012) and essential oils (Pino *et al*., 2002). Tamarinds leaves have a high content of polyphenols and are employed as antimicrobial agents (Gomathi *et al*., 2011). Some authors suggest that flavonoids and alkaloids are responsible for the antimicrobial activity of leaves extracts (Doughari, 2006). However, in a previous paper, our research group looked for a quantitative relationship between the antimicrobial activity and the polyphenols and flavonoids content in six tamarind leaves formulations and could not find a direct relationship (Escalona *et al*., 2010a)

The antimicrobial activity by direct action over the microorganism is not the only way in which a substance can't play their antimicrobial role. The activation of the complex cascade of human complement enzymes on the surface of foreign microorganisms could lead to their destruction by means of osmotic lysis (Wallport, 2001). It represents an indirect mechanism of cell lysis, in addition to the possible direct cytotoxic effect exerted by a therapeutic agent. Human complement system (composed of more than 30 plasma proteins and glycoproteins) can be activated by three different pathways, classical pathway (CP), alternative pathway (AP), and lectin pathway (LP). The influence of tamarind extracts on the complement system has been poorly investigated and just one report of tamarind fruit hydroalcoholic extract was found (Landi *et al.*, 2007).

Our previous work found that although there was a poor correlation between antimicrobial activity and flavonoid and polyphenol concentration (Escalona *et al*., 2010a); it was noteworthy that the richest extract in polyphenols (fluid extract in ethanol 70%) was the most active. This seemingly contradictory result called our attention. In order to clear up the influence of polyphenols and flavonoids in the antimicrobial activity of tamarind leaves, we proposed a chemical partitioning of the fluid extract in different polarity fractions (chemically known) to be tested in their antimicrobial activity and influence over the complement system.

MATERIALS AND METHODS

Plant material

Tamarind leaves were collected (November 2009) from a tamarind population in Santiago de Cuba (located: 20º 2´38.9´´ N y 075º 45´25.8´´ W). A voucher specimen (registered as 052216) was deposited at the [BSC] herbarium of the biology department, University of Oriente, Cuba. Collected leaves were sun dried (residual humidity below 10% by the stove method), milled (MLK, Russia), and passed across of a 5 mm of mesh light sieve.

Fluids extract preparation and fractioning

A fluid extract was prepared using ethanol 72%, 90 minutes of moistening times and percolation as preparation method (four days extraction), according to previous experiences of our group (Escalona *et al*., 2011). Divided in two parts, one was saved to be used in the microbiological analysis and quantitative procedures. The other part was fractioned by a successive liquid-liquid separation method described for flavonoid and other related substances accomplishing successive extractions with pure *n*-hexane, chloroform, ethyl acetate and *n*-butanol (Andersen and Markham, 2006). Chloroform and *n*-hexane were obtained from BDH, England, meanwhile ethyl acetate and *n*-butanol from Merck, Germany.

Phytochemical characterization

Total phenol concentrations of the extracts were spectrophotometrically estimated by the Folin–Ciocalteau reagent (Sigma, USA) (British Pharmacopeia, 2010) with total phenol content expressed as tannic acid equivalents. Flavonoids were quantified by the $AlCl_3$ method (Quettier-Deleu *et al*., 2000) and data expressed as quercetin (Fluka, Germany) equivalents. All measures were made with a CECIL CE7-200 UV-visible spectrophotometer (England).

Antimicrobial activity

Microorganism strains used in our study were *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Pseudomona aeruginosa* ATCC 27853 and *Candida albicans* CCEBI 2048 (yeast). The microorganisms were maintained at −20 °C on glycerol, in the Industrial Biotechnology Study Centre Culture Collection (CCEBI)

[http://www.aam.org.ar/cultivos_microbianos.shtml]. The plate diffusion method was used as antimicrobial test. The antibacterial activity of the tested substances was shown by a clear zone of inhibition around the application point. Reference substances were gentamycin (10 UI) and ketoconazole (30 µg). Each extract or fraction prepared at a concentration of 1 g/ml (dry leaves weight) was evaluated in a volume of 10 µl/plate, as well as the solvent control.

Determination of minimum inhibitory concentration (MIC) and minimum bactericide concentration (MBC)

The broth dilution method approved by the National Committee for Clinical Laboratory Standard (NCCLS) was followed for those extracts that exhibited some activity in the plate diffusion method in establishing the minimum inhibitory concentration (MIC). Minimum bactericide concentration (MBC) was determined as the lowest concentration in which the extract evaluated did not allow growth of organisms on the agar plate. Fraction or extract doses evaluated varied from 0.001 to 1.5 g/ml (dry leaves weight/volume).

Evaluation of complement activity

The modified hemolytic assay described by Klerx was chosen to evaluate the effects of the extracts on the complement's activity (Klerx *et al*., 1983). For this purpose, in the classical pathway, sheep erythrocytes from freshly collected blood were washed twice with buffer and sensitized with anti-sheep red blood cell antibodies (Sigma S1389, USA). Human pooled serum from healthy volunteers was used as source of complement. For the alternative pathway, the erythrocytes source was from rabbit blood (New Zeland White, PiPetit/Harlan-Ibérica) not-sensitized, washed with 0,002 M of ethyleneglycol-bis-(b-aminoethyl ether) N,N,N´,N´,-tetraacetic acid and magnesium (EGTA/Mg^{2+}). As source of complement protein, was also used human pooled serum from healthy volunteers. In the assay, the changed rate of hemolysis in the samples is compared with the uninfluenced control reactions. Quantification was spectrophotometrically made at 405 nm by a microplate reader (Benchmark Plus, Bio-Rad Laboratories Inc., USA). Data of IC_{50} values, at which the complement system activity decreases by 50% were reported; quercetin was used as a reference substance.

Statistical analysis

To examine differences between extracts, an analysis of variance (ANOVA) was performed. Significant differences between means were determined by Duncan's multiple range tests. P values < 0.05 were considered significant. SPSS 10.0 statistical software; Statsoft Inc., Tulsa, OH, USA was employed.

RESULTS AND DISCUSSION

Phytochemical characterization

Due to the fractioning method employed, ethyl acetate and *n*-butanol fractions exhibited the highest levels of phenols and flavonoids while the *n*-hexane fraction showed a very low level of phenolic. In this non-polar fraction, no flavonoid compounds were detected. Statistical differences in phenol and flavonoid concentrations were observed between all fractions, as referred Table 1. The qualitative chemical composition of those fractions was studied in preceding experiments of our group by GC/MS and HPTLC/UV-visible (Escalona *et al*., 2010b), confirming the experimental result related to the phenol and flavonoid concentration in the two last fractions. At the same time, and considering that all fractions come from the fluid extract, the recoveries values of phenols (18.03 of 18.4 µg/mL meaning a 97.7%) and flavonoids (2.34 of 2.49 meaning a 94.0%) were deemed acceptable.

Antimicrobial activity

Tamarind leaves fluid extract was active against *St. aureus, E. faecalis, B. subtilis*, *E. coli and P. aeruginosa* but inactive when faced to *S. typhimurium*, and *C. albicans*. Fractions showed some selective activity against Gram (+) but not against Gram (-) bacteria, having a different profile of sensitive strains. Nevertheless, no one equaled the original activity of the fluid extract, showing higher MIC and MBC values. On the other hand, *E. coli* and *P. aeruginosa* were sensitive to all fractions (Tables 2 and -3).

It is now well known, that antimicrobial activity in plants derives from the synthesis of phytoalexins (secondary metabolites with antimicrobial activity). Their syntheses give plants protection against bacteria and insect attack. If large quantities of phytoalexins are located throughout the entire plant, it is classified as a quantitative or immobile defense. When small amounts of toxic substances are sent to an under attack point, it is then called qualitative or mobile defense. Quantitative defense is usual in plants with a low evolutionary

level, while the second is common in plants with a superior evolutionary development. A mixture of both types of defenses should be found in plants with an intermediate position at the evolutionary scale like tamarind. Most phytoalexins are phenolic compounds by nature, but other non-phenolic compounds could play a role in this plant function (Gottlieb *et al*., 1993).

Table 1
Concentration (µg/mL) of total polyphenols and flavonoids in a tamarind leaves 70% fluid extract and its four fractions.

Fraction or extract	Polyphenols		Flavonoids	
	Concentration (µg/mL)	%	Concentration (µg/mL)	%
Fluid extract 70% ethanol	18.40[a]	100	2.49[a]	100
n-hexane fraction	0.76[e]	4.13	0.00	0.00
chloroform fraction	1.38[d]	7.50	0.18[d]	7.23
ethyl acetate fraction	5.28[c]	28.70	0.89[c]	35.74
n-butanol fraction	10.60[b]	57.61	1.27[b]	51.00

Different letters mean statistical differences. Duncan's multiple range tests. *P* values < 0.05

Table 2
Microbial susceptibility to 10 µl (1 mg/ml) of the tamarind leaves extract or its fractions; gentamycin (10 UI) and ketoconazole (30 µg).

Species	FE. 70%	n-hex	Chlor	E. acet	n-but	Gent	Keto
S. aureus	+	+	+	-	-	+	-
E.faecalis	+	-	-	+	+	+	-
B. subtilis	+	-	+	+	+	+	-
P. aeruginosa	+	+	+	+	+	+	-
E. coli	+	+	+	+	+	+	-
S. typhimurium	-	-	-	-	-	+	-
C. albicans	-	-	-	-	-	-	+

FE. 70%→ Fluid extract 70% ethanol. **n-hex→ n-hexane fraction**
Chlor→ Chloroform fraction **E. acet→ Ethyl acetate fraction**
n-but→ n-butanol fraction **Gent→ Gentamycin**
Keto→ Ketoconazole

A possible work hypothesis could be: if the antimicrobial activity depended on the total amount of phenol and/or flavonoid concentration, it can be an expression of a quantitative type defense; indeed the antimicrobial activity should be detected into the fractions with higher phenol/flavonoid quantities. Otherwise, if special kinds of phenols and/or flavonoids are the enough toxic to exhibit the antimicrobial activity in low concentrations (a characteristic behaviour of a qualitative defense); the activity measured not necessarily going to be determinate in the high phenol/flavonoid concentrated fractions. This last possibility can be affected by non-phenolic metabolites also involved in the antimicrobial activity. Nevertheless these non-phenolic metabolites are characteristic in a qualitative defense type. Quantitative defense allowed for mathematical correlation then, this work hypothesis could lead us to define if it is possible to wait for a good activity/concentration relationship.

The experiment results expose three different behaviours when fractions were tested. When assayed

against *S. aureus*, the active fractions were those with lower levels of phenols and flavonoids (see Tables 1 and 3). The absence of flavonoids in *n*-hexane fraction puts aside the activity of these compounds over *S. aureus*. By this way, the activity measured can be interpreted as the result of the action of a very toxic phenol substance, or a result of the antimicrobial activity related with non-phenol compounds as fatty acids which are present in the *n*-hexane and chloroform active fractions (Escalona *et al*., 2010b), and has been extensively well-informed as active against this bacterium (Thormar & Hilmarsson, 2007). In any case, both possibilities result a qualitative defence type that cannot be expressed by linear correlation activity/concentration studies.

Table 3
MIC and MBC values (expressed as dry leaves weight/volume in g/mL) of the tamarind fluid extract and its fractions when tested with different microorganisms.

Species	FE. 70% ethanol		n-hexane F.		Chloroform F.		Ethyl acetate F.		n-butanol F.	
	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC
S. aureus	0.75	0.094	1.5	0.38	1.5	0.38	-	-	-	-
E. faecalis	0.75	0.047	-	-	-	-	1.5	0.75	1.5	0.75
B. subtilis	0.375	0.094	-	-	>1.5	0.75	1.5	0.38	0.75	0.19
P. aeruginosa	1.5	0.187	>1.5	0.75	>1.5	0.75	>1.5	0.38	>1.5	0.38
E. coli	1.5	0.187	1.5	0.75	>1.5	0.75	>1.5	0.75	1.5	0.75
S. typhimurium	-	-	-	-	-	-	-	-	-	-
C. albicans	-	-	-	-	-	-	-	-	-	-

On the opposite side, when the fractions are faced with *E. faecalis* and *B. subtilis* the more polar fractions with higher phenol/flavonoid concentration were more active, even with a change of MIC values in the case of *B. subtilis* (see Table 3). As same as the previous paper (Escalona *et al*., 2010a), in order to correlate the influence of total phenols and flavonoids on the activity measured; we estimate the real concentration in which these metabolites were present in the MIC and MBC previously calculated. This estimation consists in multiplying the phenol/flavonoid concentration value by the MIC or MCB determinate. Once again, only for *B. subtilis* we found similar values of total phenol concentration in the all MIC (1.73, 1.04, 1.98 and 1.99 μg/mL) and MBC (6.90, 7.92 and 7.95 μg/mL) calculated, meaning a particular susceptibility of this bacterium when faced to phenolic compounds. In fact, only over this bacterium, a linear correlation gives an appreciable value (r^2 = 0.82). The rest of the estimations render dissimilar values between the extracts, and were considered as a low influence of these compounds on the activity.

For Gram (-) bacteria a non selective activity was observed when all fractions were active against *E. coli and P. aeruginosa*. It seems like tamarind leaves produce compounds active against this bacteria but chemically different. This observation joined to the fact that in all cases the MBC and MIC calculated for the fluid extract were lower than those calculated for the fractions; suggest that the complete pool of compounds present in the fluid extract are more effective in their antimicrobial activity, and at the same time, that phenol and flavonoid compounds are not the only able to exhibit antibacterial activity.

Complement activity

In the classical pathway, the tamarind fluid extract, as well as the *n*-butanol and the ethyl acetate fractions inhibited complement activity in a concentration-dependent manner as shown in Figure 1. The IC_{50} were 31.05 μg/mL, 33.65 μg/mL and 55.8 μg/mL for

n-butanol fraction, ethyl acetate fraction and the fluid extract, respectively. Under the same experimental conditions, the IC_{50} for quercetin was 27.15 μg/mL. The *n*-hexane and the chloroform fractions had no effect on the complement classical pathway. Neither the fluid extract nor any of its fractions had effect over the alternative pathway.

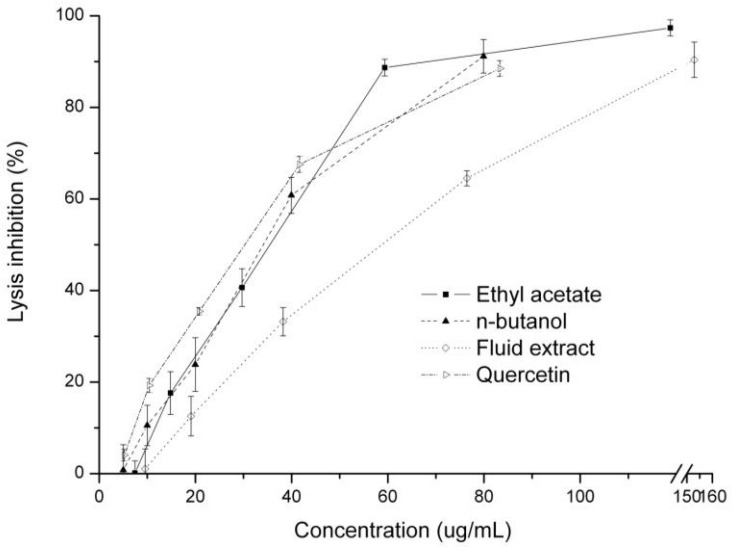

Figure 1
Lysis inhibition of *Tamarindus indica* L fluid extract and its fractions (Ethyl acetate and n-butanol), expressed as percentage.

The study on the antimicrobial effect mediated by the classical pathway complement activation demonstrates that tamarind fluid extract, as well as the *n*-butanol and the ethyl acetate fractions are inhibitors of the of human complement activation *in vitro*. This means that the tamarind leaves components extracted in those fractions (mainly phenols and flavonoids) didn't assist by this way the antimicrobial activity, on the contrary, acts as inhibitors of the immune system. From them, the *n*-butanol and the ethyl acetate fractions showed the most potent inhibitory activity against complement-induced haemolysis *via* the classical pathway with IC_{50} values close to that obtained for quercetin.

The presence of hydroxyl groups in polyphenols and flavonoids, could be critical for the anti-complementary action of tamarind leaves extracts. The hydroxyl groups of polyphenols and flavonoids may act as acceptors for C3b and C4b. It has previously been reported that hydroxylated polymers such as cellulose or unmodified dextran (Crepon *et al*., 1987) interact with the labile binding site of nascent C3b and thus, having true inhibitory properties towards the convertasa formation.

On the other hand, the crude hydroalcoholic extract and its fractions had no effect on the alternative pathway. This "apparent activity contradiction" has been extensively reported for natural products, even for tamarind a fruit extracts (Landi *et al*., 2007); due to the known chemical complexities of natural extracts. Although results do not support a major role for the complement system on the antimicrobial effect of tamarind leaves extracts, tamarind formulations promise to have a good potential as anti-complementary and anti-inflammatory agents.

CONCLUSIONS

Tamarind leaves have worldwide use as antimicrobial agents in traditional medicine. Previous works and literature pointed to phenols and flavonoids as the source for this activity, but no quantitative relationship has been found between these metabolites and the antibacterial activity of the leaves. In this paper we try to explore the influence of these compound in the most

active preparation (ethanol 70% fluid extract), but still not relevant correlation was detected between phenols and flavonoids content and the antimicrobial activity in their different fractions. Neither, complement activation was the mechanism through which tamarind leaves play an antimicrobial role. In consequence, we propose that phenols and flavonoids concentration could be important for some kind of bacteria but not for the all tested; which could be sensible to other non-phenolic types of metabolites. This proposal, could justify the poor quantitative correlation between these compounds and the antimicrobial activity observed in the previous works, leaving room for the antimicrobial role of other secondary metabolites.

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