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Scientific Research and Essays

Full Length Research Paper

Honey activity on the proliferation and migration of oral squamous cell carcinoma: *In vitro* and bioinformatic analysis

Eliane Macedo Sobrinho Santos[1*], Hércules Otacílio Santos[1], Juliana Rezende Sá Miranda Gonçalves[1], Anna Christina Almeida[2], Igor Viana Brandi[2], Alex Sander Rodrigues Cangussu[3], Janainne Nunes Alves[1], Sabrina Ferreira de Jesus[4], Ricardo Jardim Neiva[1], Kattyanne de Souza Costa[5], André Luiz Sena Guimarães[4] and Lucyana Conceição Farias[4]

[1]Federal Institute of Northern Minas Gerais, Campus Araçuaí, Minas Gerais, Brazil.
[2]Institute of Agrarian Sciences of UFMG, Campus Montes Claros, Minas Gerais, Brazil.
[3]Federal University of Tocantins, Campus Gurupi, Tocantins, Brazil.
[4]Department of Dentistry, Universidade Estadual de Montes Claros, Montes Claros, Brazil.
[5]MSD Site Montes Claros, Minas Gerais, Brazil.

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This study aimed to investigate, in oral squamous carcinoma cells (OSCC), the antineoplastic effect of *Apis mellifera* honey produced from Aroeira and wildflower, in the region of Jequitinhonha River Valley, Brazil. Furthermore, we investigate the molecular mechanism of honey performance through bioinformatic analysis. Honey and OSCC-related gene list were screened using GeneCards. The online tool STRING was used to identify the potential interaction networks of protein products of these genes. The genes with the highest weighted number of links (WNL) were called leader genes. GO and KEGG pathway analysis was performed to classify the genes. We use a search tool STITCH for interactions of chemicals-proteins. The chemical characterization of honey and its antiproliferative and antimigratory effects in SCC-9 cell lines was evaluated *in vitro*. The analysis of the interaction network obtained from the STRING database indicated by the following leader genes: TNF, IL6 and HDAC1. GO analysis for the honey, and OSCC-related proteins revealed that leader genes-regulated cancers genes were markedly enriched in some functions. Through the chemical characterization, the bioactive classes: steroids/triterpenoids, flavonoids, saponins, alkaloids, and tannins were identified. Polyphenols act in activating CASP3 and TP53 and inhibit the action of DNMT1. The honey produced from Aroeira and wildflower reduced proliferative phenotype and migration of OSCC cells.

Key words: Aroeira honey, *Myracrodruon urundeuva*, wild honey, *Apis mellifera*, therapeutic, Jequitinhonha River Valley.

INTRODUCTION

Honey is a product that presents characteristics not only of the bees that originate it but also has botanico-geographical characteristics from the geographic area which it was produced. The honey processed from the nectar of flowers and/or saccharinic secretions of other species carries inheritance of the plant species of origin. Among these inheritances are some bioactive properties resulting from secondary metabolism in plant species

(Anand et al., 2018; Deng et al., 2018). The region of Jequitinhonha River Valley, located in northeastern of Minas Gerais State, Brazil, is suitable for beekeeping, as it has biodiversity of plant species allowing the harvesting of honey during most of the year (Miles et al., 2006).

Oral Squamous Cell Carcinoma (OSCC) represents a worldwide public health problem, not only due to the high prevalence rates but mainly due to the survival rates, morbidity, and mortality associated with the disease (Petersen, 2005). The great knowledge of the science about the etiopathogenesis of the disease does not, however, correspond to the improvement in the prognosis and treatment of the individuals affected (Day et al., 2003; Bramer et al., 2009).

Sudies have shown that honey has excellent therapeutic effects since it presented important antineoplastic and cytotoxic properties in several neoplastic cells (Swellam et al., 2003; Orsolic et al., 2005), although its role is not yet fully elucidated. However, no study has so far explored the possibility of the performance of the Aroeira and wild honey of the Jequitinhonha River Valley to the therapeutic effect in the OSCC.

Thus, this study aimed to investigate oral squamous carcinoma cells of antineoplastic effect of *Apis mellifera* honey produced from Aroeira (*Myracrodruon urundeuva*) and wildflower, in the region of Jequitinhonha River Valley, located in northeastern of Minas Gerais State, Brazil. Furthermore, we investigate the molecular mechanism of honey performance through bioinformatic analysis.

In this context, this study is justified by a new research approach about the antineoplastic potential of substances contained in the honey, aiming to mitigate the invasive behavior of the disease.

MATERIALS AND METHODS

Data and sample collection

Honey samples were collected from the honey of *A. mellifera* harvested at the time where the flowering of Aroeira predominated, being the predominance of pollen in the samples belonging to *M. urundeuva* (Anacardiaceae-Aroeira). In the samples of honey of wildflowering, there is a great diversity of plants supplying nectar for the production, including the Aroeira with differentiated flowering.

Samples of honey from Aroeira and wildflowers, as well as information about them, were extracted from the database of AAPIVAJE - Association of Beekeepers of Vale do Jequitinhonha, located in the municipality of Turmalina city, located in Jequitinhonha River Valley, Brazil, from March to September 2017. The microscopic, microbiological and physicochemical characterization of the honey was previously obtained by the team (data not yet published).

Detection of chemical compounds in honey

The first extraction of phenolic compounds from honey followed the method described by Ferreres et al. (1994), with some modifications. To prepare, about 50 g honey extracts were weighed, which were mixed with 250 mL of distilled water.

The resulting solution was stirred with magnetic stirrer at room temperature until complete dissolution, thereafter the pH was adjusted to 2.0 with concentrated HCl. The flowable sample was then filtered through cotton to remove possible solid particles. The methods of qualitative investigation of the following compounds are described below.

Phytochemical characterization was performed according to the methodologies of Matos (1997) and Ugaz (1994).

Steroids/triterpenoids

The steroid/triterpenoid tests were performed by the Lieberman-Burchard reaction where 2 mL of the extract was used, mixed with 2 mL of chloroform, thereafter the chloroform solution was filtered dropwise on a cotton funnel. In a test tube, 1 mL of acetic anhydride was added, gently shaken, and three drops of concentrated H_2SO_4 was added carefully, gently stirring and observing color development. The evanescent blue color followed by green indicates the presence of steroids/triterpenoids respectively.

Flavonoids

For the identification of flavonoids, the cyanidin or Shinoda test (concentrated HCl and magnesium) was performed where 2 mL of extract was added to approximately 0.5 cm of magnesium on tape with 2 mL of concentrated hydrochloric acid. The end of the reaction is due to the end of effervescence. The appearance of coloration that varies from brown to red indicates the presence of flavonoids in the extract.

Tannins

For the identification of tannins, 2 mL of the extract was placed in a test tube, and three drops of an alcoholic solution of $FeCl_3$ were added with strong shaking. In the test, the formation of a precipitate of blue tint indicates the presence of hydrolyzable tannins, and green, the presence of condensed tannins.

Saponins

In 2 mL of the extract were added 2 mL of chloroform and 5 mL of distilled water shortly after the filtration was put into a test tube. Thereafter the solution was stirred permanently for 3 min, and frothing was observed. Persistent and abundant foam indicates the presence of saponin.

Alkaloids

30 mL of extract was added, 5 mL of HCl (10%) was added and the mixture heated for 10 min. The material was cooled, filtered, divided into two test tubes and a few drops of the recognition

*Corresponding author. E-mail: elianemsobrinho@hotmail.com. Tel: +55 (038) 98819-9270.

reagents: Mayer and Wagner. Slight turbidity or precipitate (purple, white to cream and brown) evidences the possible presence of them.

Evaluation of antiproliferative and antimigratory potential of the SCC-9 cell

In order to verify the antineoplastic potential of honey from different flowering plants, the antiproliferative and antimigratory effect in SCC-9 cell lines was evaluated.

Cell culture

An immortalized cell line of squamous cell carcinoma of the mouth SCC-9, commercially acquired (ATCC, USA) was used. This was stored in ultra-freezer at -80°C and cryopreserved in a specific solution.

Defrosting and cell culture

Cells were thawed in a 37°C water bath and cultured in culture medium specific to each cell line, as recommended by the supplier. The steps for thawing and cell culture were performed according to specific protocols of the Oral Health Research Laboratory of the State University of Montes Claros (Sobrinho et al., 2017). The cells were kept in an oven until reaching subconfluence of 50 to 60%, when they were categorized in the experimental groups, and submitted to different concentrations of treatments with honey, and analyzes as described below. All experiments were performed in triplicates and at three experimental times, to guarantee the reliability of the results.

Treatment with Aroeira and wild honey of the Jequitinhonha River Valley

Cells were treated with honey at a predetermined concentration for 24 h by standardizing the dose/time response curve (Supplementary Material Figure 1). The treatments were carried out on semiconfluent cultures of SCC-9 maintained in 12-well plates. The analyzes were performed after the predetermined time for treatment.

Cell proliferation assay

In order to test the biological activity of honeys, evaluating their ability to inhibit or stimulate cell growth, the cell quantification method was used in Neubauer's chamber (Freshney, 1994). The number of cells per ml of a suspension when counted was obtained by the equation:

$$\text{Number of cells / ml} = \frac{\text{Total number of cells x dilution factor x 10,000}}{\text{Number of counted quadrants}}$$

Cell migration test

Cell migration was evaluated by the wound-healing assay according to Chan et al. (2008). Briefly, the cells were grown in 6-well culture plates until reaching confluency. Subsequently, the culture medium was removed, and a scarification was produced on the cell monolayer using a 200 µl tip. Cells were washed with PBS solution and incubated at 37°C in the oven with complete culture media in the presence or absence of a specific concentration of honey.

At the indicated times (0 and time 24 h), the phase contrast images of specific sites of the damage produced were captured. The images were then analyzed, and a measure of the area of the damage (in µm) was performed through the specific software evaluating how much the cells were able to advance in search of the confluence.

The migration quantification was performed by the percentage of the area covered in the culture time determined in relation to the time zero hour. Data were expressed as the difference between the coated surface in response to treatment with honey and the basal condition in the absence of honey (% surface covered treatment - basal covered surface).

Bioinformatic analysis

Honey and OSCC-related genes list were screened using GeneCards (https://www.genecards.org). The online tool STRING (http://string-db.org) was used to identify the potential interaction networks of protein products of these genes. The network was expanded three times. The minimum required interaction score was medium confidence (0.4) and max number of interactors showed no more than ten interactors in 1st and 2nd shells. The genes with the highest weighted number of links (WNL), called leader genes, were identified through the sum of the following active interactions sources: Textmining, Experiments, Databases, Co-expression, Neighborhood, Gene Fusion, and Co-occurrence. GO and KEGG pathway analysis was performed to classify the genes. The GO analysis demonstrated that the genes were enriched in some biological processes, molecular function and cellular component (Sheng et al., 2017).

For the design and interpretation of these studies, the context of the chemicals and proteins needs to be considered. Therefore, we use a search tool for interactions of chemicals-proteins (https://stitch.embl.de) both as a large-scale, downloadable database of interaction data and as an interactive web tool for the exploration of interaction networks. The input descriptors in the STITCH platform were: Steroids/Triterpenoids, Flavonoids, Saponins, Alkaloids, Tannins and Polyphenols (Kuhn et al., 2010).

Statistical analysis

All the data collected were scanned in a statistical program SPSS®, version 13.0, for Windows. The statistical analysis was carried out to analyze the association between the treatments of the cells and the phenotypic behavior of the same ones related in the proposal. The statistical tests were selected according to the characteristics of the samples and the distribution of the variables. The level of significance was set at 5% ($p < 0.05$).

RESULTS

Network of honey and OSCC-related genes

Analysis of honey and OSCC-related genes in GeneCards identified 27 genes (Table 1). Protein-protein interaction networks of 27 genes were constructed using the STRING database analysis (Figure 1A) ($p < 1.0^{-16}$). The TNF, IL6 and HDAC1 genes showed a large number of interactions. The STITCH network automatically extracted the targets of polyphenols (Figure 1B), which

Table 1. Honey and OSCC-related genes list screened using GeneCards.

Symbol	Description	GIFtS	GC id	Score
MMP9	Matrix Metallopeptidase 9	63	GC20P046008	12.61
CAT	Catalase	61	GC11P034460	4.63
IL6	**Interleukin 6**	**59**	**GC07P022765**	**4.52**
TNF	**Tumor Necrosis Factor**	**64**	**GC06P031673**	**4.48**
CYP2E1	Cytochrome P450 Family 2 Subfamily E Member 1	55	GC10P133520	4.33
GSR	Glutathione-Disulfide Reductase	59	GC08M030655	4.02
LY96	Lymphocyte Antigen 96	53	GC08P073991	3.85
CRP	C-Reactive Protein	56	GC01M159682	2.76
HDAC9	Histone Deacetylase 9	54	GC07P018179	2.73
NAT2	N-Acetyltransferase 2	52	GC08P018391	2.67
TERT	Telomerase Reverse Transcriptase	61	GC05M001253	2.66
CYP2D6	Cytochrome P450 Family 2 Subfamily D Member 6	61	GC22M042131	2.61
HDAC1	**Histone Deacetylase 1**	**60**	**GC01P032260**	**2.47**
IFNG	Interferon Gamma	58	GC12M068064	2.38
LTA	Lymphotoxin Alpha	50	GC06P031572	2.20
G6PD	Glucose-6-Phosphate Dehydrogenase	59	GC0XM154531	2.09
HDAC2	Histone Deacetylase 2	61	GC06M113933	1.84
ODC1	Ornithine Decarboxylase 1	55	GC02M010432	1.76
PPARG	Peroxisome Proliferator Activated Receptor Gamma	64	GC03P012328	1.76
ANXA1	Annexin A1	59	GC09P073151	1.47
CSF1	Colony Stimulating Factor 1	54	GC01P109911	1.42
XDH	Xanthine Dehydrogenase	59	GC02M031294	1.32
NR1H2	Nuclear Receptor Subfamily 1 Group H Member 2	57	GC19P050329	1.18
RXRA	Retinoid X Receptor Alpha	58	GC09P134317	1.07
NAT1	N-Acetyltransferase 1	54	GC08P018178	0.84
LOX	Lysyl Oxidase	54	GC05M122063	0.43
PPP2CA	Protein Phosphatase 2 Catalytic Subunit Alpha	56	GC05M134194	0.35

Source: GeneCards.
Leader genes are shown in bold.

are compounds found in the chemical composition of honey. The interaction between polyphenols and CASP3, AKT1, CYP1A2, DNMT1, IL8, MAPK8, NOS3, TP53, TTR, VEGFA genes occurs expressively with a score above 0.9 (Table 2). The analyzes also showed that polyphenol acts in activating CASP3 and TP53 and inhibit the action of DNMT1, according to data predicted in Table 3. A subnetwork was elaborated from these genes and those genes that presented a large number of interactions in the STRING database (Figure 1C). Significant direct or indirect interactions between these genes were observed.

Leader genes serve a key function of honey in the OSCC

The analysis of the interaction network obtained from the STRING database indicated the following leader genes: TNF, IL6 and HDAC1 genes. It can be seen that the leading genes occupy the cluster with the highest WNL values (Figure 2A). Statistical analyzes confirm and validate such findings (Table 4). Leader genes exert a great influence on the interaction network since they have a large number of interactions.

GO analysis for the honey and OSCC-related proteins revealed that leader genes-regulated cancers genes were markedly enriched in a number of functions, including regulation of cellular metabolic process, regulation of protein metabolic process, regulation of cellular protein metabolic process (Biological Process) (Figure 2C), protein binding, protein deacetylase activity, protein dimerization activity (Molecular Function) (Figure 2D), histone deacetylase complex, NuRD complex, protein phosphatase type 2A complex (Cellular Component) (Figure 2E). The results indicated that leader genes are involved in cancer cell proliferation and migration. In addition, KEGG pathway analysis revealed that the honey and OSCC-related genes were markedly enriched in Chagas disease, PI3K-Akt signaling pathway, Hepatitis C, NF-kappa B signaling pathway, and Transcriptional misregulation in cancer (Figure 2F).

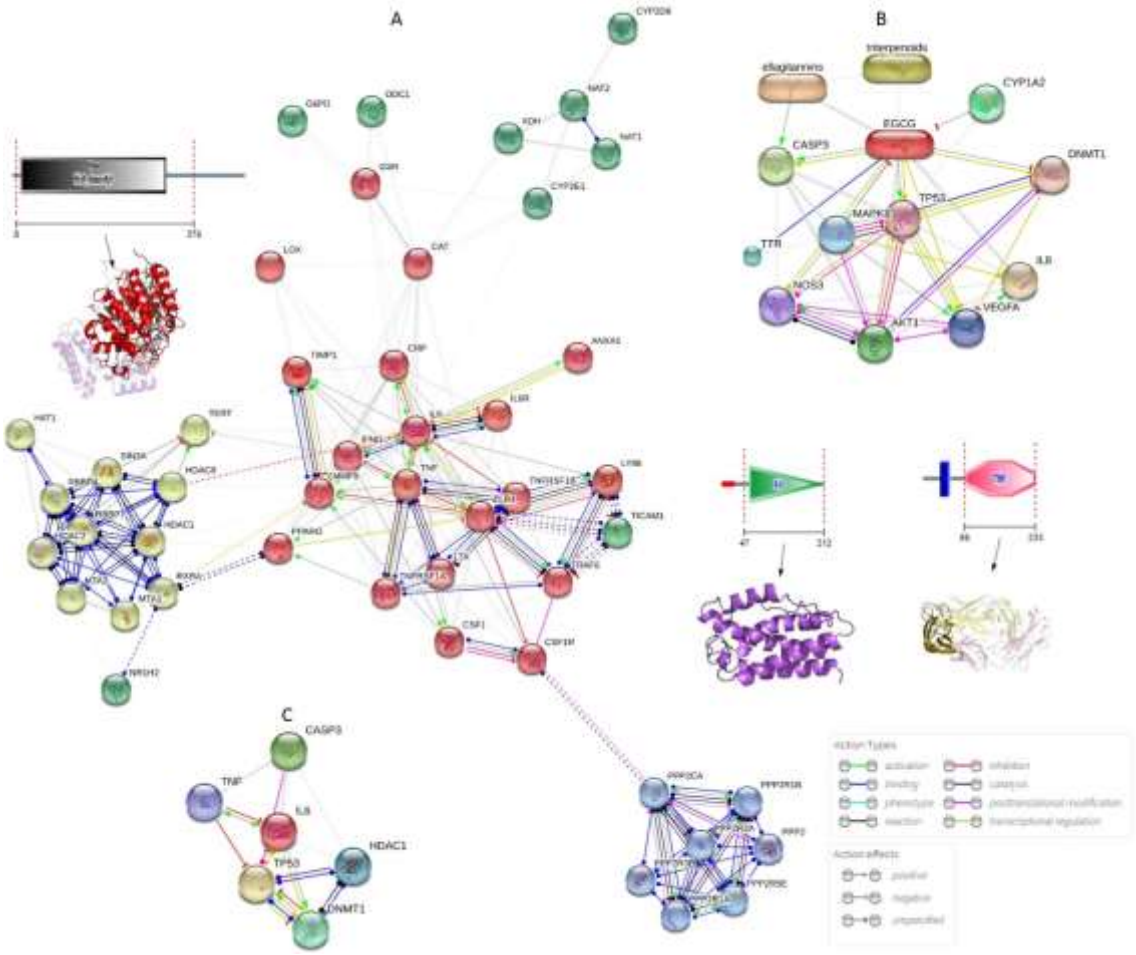

Figure 1. Interaction network of honey and OSCC-related genes were analyzed using the STRING database. The interactions of 47 honey and OSCC-related genes (A). Interaction network of polyphenols-related genes was analyzed using the STITCH database. The interactions of 10 polyphenols-related genes (B). Interaction network of leader genes and polyphenols-related genes (C).

Table 2. Combined score of interaction between genes and chemical compounds present in honey.

Node1	Node2	Score	Node1	Node2	Score	Node1	Node2	Score	Node1	Node2	Score
AKT1	CASP3	0.978	EGCG	AKT1	0.964	MAPK8	EGCG	0.961	TP53	TTR	0.414
AKT1	DNMT1	0.950	EGCG	CASP3	0.967	MAPK8	IL8	0.909	TP53	VEGFA	0.961
AKT1	EGCG	0.964	EGCG	CYP1A2	0.963	MAPK8	NOS3	0.675	TTR	EGCG	0.961
AKT1	IL8	0.700	EGCG	DNMT1	0.957	MAPK8	TP53	0.999	TTR	TP53	0.414
AKT1	MAPK8	0.481	EGCG	IL8	0.954	MAPK8	VEGFA	0.890	VEGFA	AKT1	0.982
AKT1	NOS3	0.999	EGCG	MAPK8	0.961	NOS3	AKT1	0.999	VEGFA	DNMT1	0.850
AKT1	TP53	0.975	EGCG	NOS3	0.958	NOS3	CASP3	0.518	VEGFA	EGCG	0.959
AKT1	VEGFA	0.982	EGCG	TP53	0.958	NOS3	EGCG	0.958	VEGFA	IL8	0.942
CASP3	AKT1	0.978	EGCG	TTR	0.961	NOS3	IL8	0.568	VEGFA	MAPK8	0.890
CASP3	EGCG	0.967	EGCG	VEGFA	0.959	NOS3	MAPK8	0.675	VEGFA	NOS3	0.960
CASP3	MAPK8	0.843	EGCG	ellagitannins	0.913	NOS3	TP53	0.913	VEGFA	TP53	0.961
CASP3	NOS3	0.518	EGCG	triterpenoids	0.463	NOS3	VEGFA	0.960	Ellagitannins	CASP3	0.728
CASP3	TP53	0.914	IL8	AKT1	0.700	TP53	AKT1	0.975	Ellagitannins	EGCG	0.913
CASP3	ellagitannins	0.728	IL8	EGCG	0.954	TP53	CASP3	0.914	Ellagitannins	Triterpenoids	0.400

Table 2. Contd.

CYP1A2	EGCG	0.963	IL8	MAPK8	0.909	TP53	CYP1A2	0.430	Triterpenoids	EGCG	0.463
CYP1A2	TP53	0.430	IL8	NOS3	0.568	TP53	DNMT1	0.985	Triterpenoids	Ellagitannins	0.400
DNMT1	AKT1	0.950	IL8	TP53	0.645	TP53	EGCG	0.958	DNMT1	VEGFA	0.850
DNMT1	EGCG	0.957	IL8	VEGFA	0.942	TP53	IL8	0.645	MAPK8	CASP3	0.843
DNMT1	TP53	0.985	MAPK8	AKT1	0.481	TP53	MAPK8	0.999	TP53	NOS3	0.913

Analyzes extracted from the STITCH database. The interaction between polyphenols and genes appears in red.

Table 3. Predicted Functional Partners.

Proteins	Activation	Inhibition	Binding	Expression	Score
CASP3	x			x	0.967
AKT1	x				0.964
CYP1A2	x	x			0.963
TTR		x	x		0.961
MAPK8	x	x			0.961
VEGFA		x		x	0.959
NOS3	x	x		x	0.958
TP53	x			x	0.958
DNMT1				x	0.957
IL8	x	x			0.954

Analyzes extracted from the STITCH database.

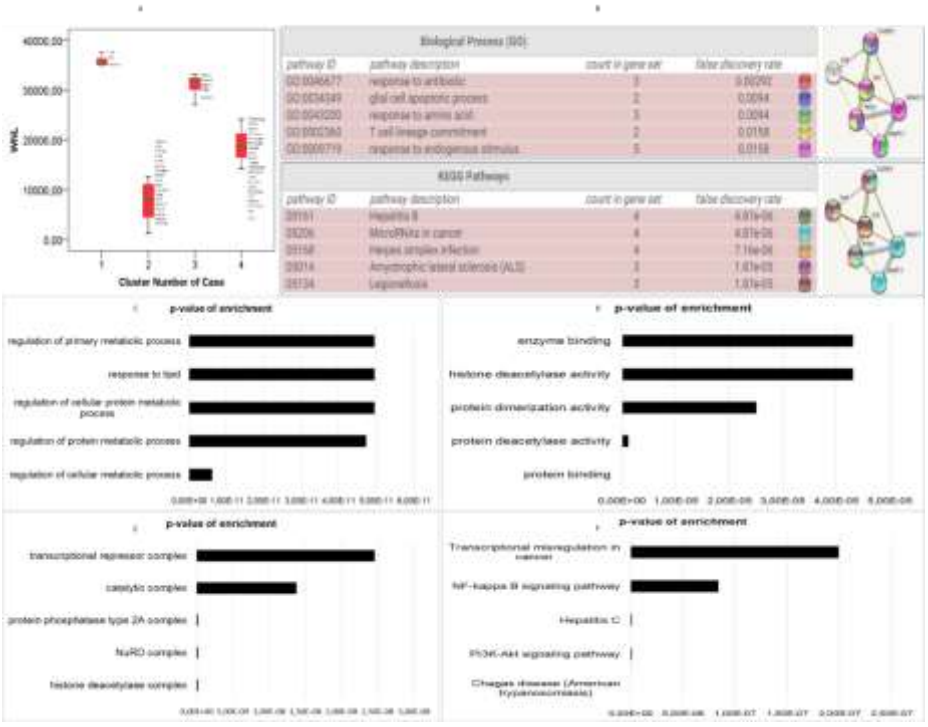

Figure 2. Gene clusters. Genes leaders belong to the cluster with larger WNL (A). Gene Ontology analysis of interaction network of leader genes and polyphenols-related genes (B)*. Analysis of honey and OSCC-related genes. Gene Ontology analysis: biological process (C), molecular function (D), cellular component (E) and Kyoto Encyclopedia of Genes and Genomes pathway (F). * Data extracted from STRING database.

Table 4. Validation analysis of gene clusters.

Clusters			Number			
			1	2	3	4
Initial cluster centers						
WNL			37651,00	1278,00	29998,00	27079,00
Final cluster centers						
Clusters			1	2	3	4
WNL			35948,00	7431,94	30948,40	19138,24
ANOVA						
Data	Cluster		Error		F	Sig.
	Mean square	df	Mean square	df		
WNL	1.265E9	3	1.163E7	43	108.752	0.000

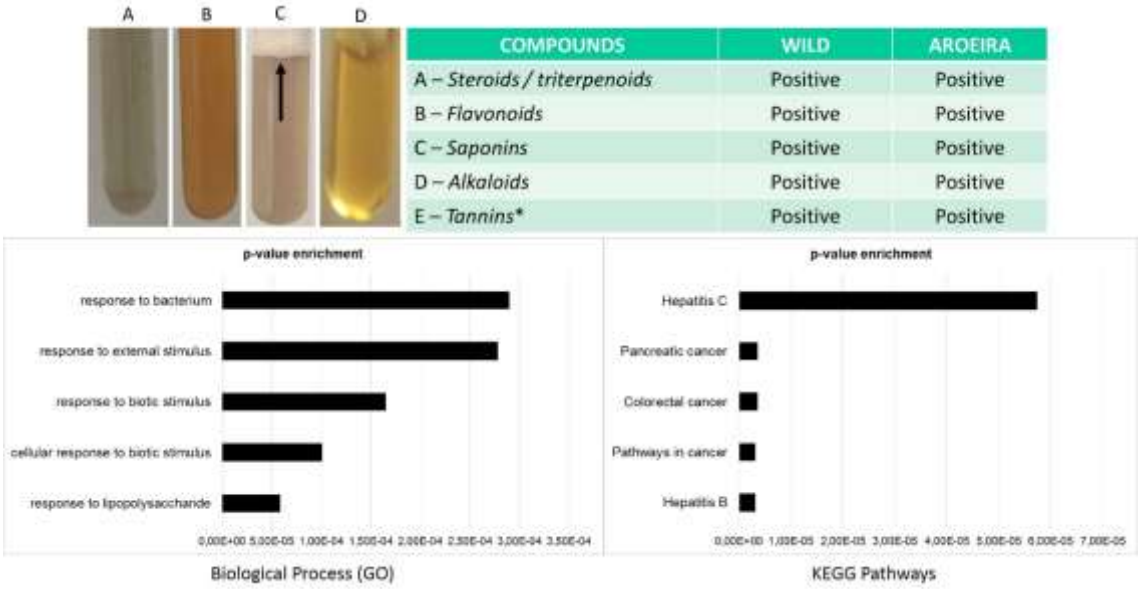

Figure 3. Polyphenols analysis in honey. A qualitative investigation of chemical compounds of aroeira and wild honey. *Not visible in the photo. Foam indicated by the arrowhead. A positive test indicates the presence of compounds. Analysis of polyphenols-related genes. Gene Ontology analysis: biological process and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway.

Notably, in the subnetwork formed from the leading genes and genes regulated by the polyphenols it is possible to notice that the genes participate in the following biological processes: response to antibiotic, glial cell apoptotic processes, response to amino acid. KEGG pathway analysis revealed that the genes were markedly enriched in Hepatitis B, microRNAs in cancer, herpes simplex infection (Figure 2B).

Chemical characterization of honey

The quality of the honey depends on its chemical composition and floral origin. The content of chemicals compounds is strongly affected by their floral origin, geographical and climatic characteristics of the place. For these reasons, the identification and quantification of chemicals compounds of honey are of great interest. As shown in Figure 3, the following classes of compounds were detected in honey samples: steroids / triterpenoids, flavonoids, saponins, alkaloids and tannins. These compounds may be associated with the therapeutic activities of honey. The bioactive classes found are derived from the secondary metabolism of the species and are associated with the environmental stress to which the species were exposed. Honey, as a product that has botanical characteristics through the contact of the bees with the local flora, carries vegetable heritages,

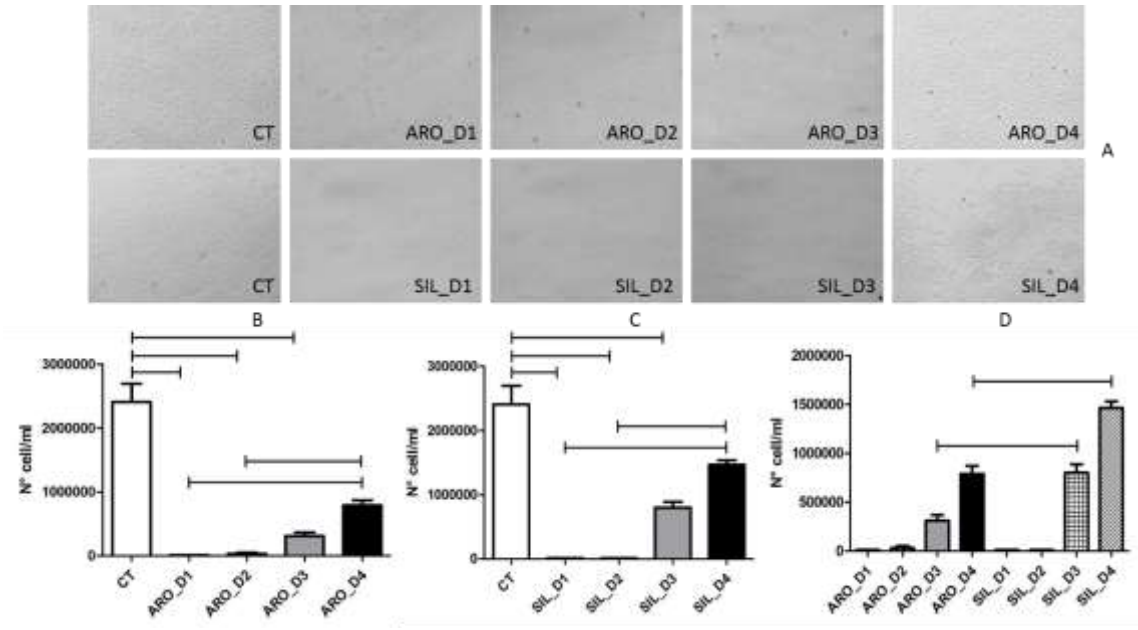

Figure 4. Cell proliferation assay. (A) Morphology of the cells of the control groups and treated with aroeira or wild honey. (B) Change in a number of cells after treatment with aroeira honey. (C) Change in a number of cells after treatment with wild honey. (D) Comparison of the number of cells after treatment with aroeira honey and wild honey. CT, Control; ARO, Aroeira honey; SIL, Wild Honey. Significance was determined using the ANOVA ONE WAY Statistical Test (Symbol ⊓ : P <0.05 control vs. aroeira honey or wild honey).

resulting in a unique product according to the region where it is produced. On the other hand, the effect of the bioactive compounds can occur alone or synergistically, causing the honey from the Jequitinhonha River Valley to have exclusive antitumor effects.

GO analysis for the polyphenols-related proteins revealed that cancers genes were markedly enriched in some functions, including biological processes, such as response to lipopolysaccharide, cellular response to biotic stimulus, response to biotic stimulus, response to external stimulus and response to bacterium. Besides that, the KEGG pathway analysis revealed that the polyphenols-related genes were markedly enriched in Hepatitis B, pathways in cancer, colorectal cancer, pancreatic cancer and Hepatitis C (Figure 3).

Effect of honey on SCC-9 cell proliferation

After 24 h of treatment, the cells without treatment with honey (Control Group) presented a polygonal shape, which is considered the phenomenon of normal cell growth. However, when the cells were treated with 25, 6.25 and 1.56% of honey, both Aroeira and wild, for 24 h, the cells were rounded and had an abnormal morphological appearance (Figure 4A). Cells with vacuolized membranes could also be recognized at these same concentrations. At the concentration of 0.39% of both kinds of honey, the little alteration was observed in the morphology of the cells when compared with the cells of the control group (Figure 4A).

It is important to emphasize the great presence of cellular debris in the group of cells treated with wild and Aroeira honey. These debris were not visualized in the control group. When the cells were treated with the different concentrations of honey, both Aroeira and wild, for 24 h, a reduction in the number of cells was observed when compared to the control group. In the group of cells treated with Aroeira and wild honeys, a large number of dead cells were observed, which corresponds to the reduction of proliferation in these cell groups (Figure 4B and 4C).

Although a decrease in the number of cells treated with all honey concentrations was observed, when compared with the control group, interestingly, at concentrations of 1.56 and 0.39%, it can be seen that the number of cells presented reduced in Aroeira honey treatment when compared to the group treated with wild honey (Figure 4D).

Effect of honey on the migration of SCC-9 cells

Figures 5A and 5B illustrate the migration behavior of the cells treated with Aroeira honey to 25% and Aroeira and wild honey to 1.59%, respectively.

Statistically, it can be verified that the reduction in the migration capacity of the cells treated with the honeys

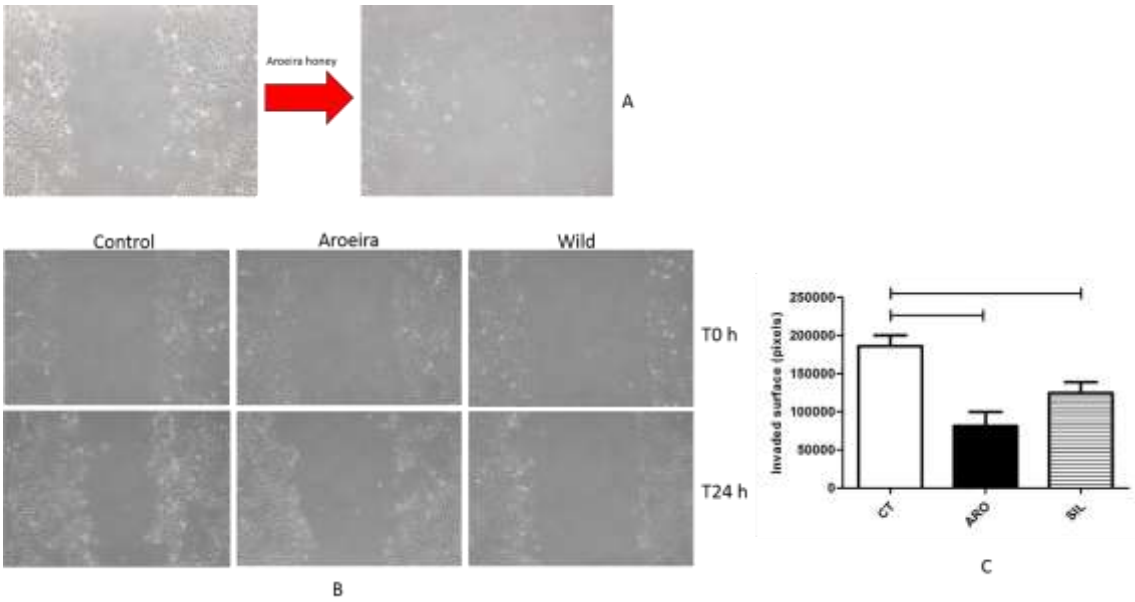

Figure 5. Cell migration assay. (A) Representative photographs of cell migration in the wound healing process after 25% honey treatment. (B) Representative photographs of the cellular migration in the process of "wound healing" after treatment with 1.59% of honey of aroeira or wild. (C) Surface invaded by untreated cells and treated with aroeira or wild honey at a concentration of 1.59%. CT, Control; ARO, Aroeira honey; SIL, Wild Honey Significance was determined using the ANOVA ONEWAY Statistical Test (Symbol ⊓ : P <0.05 control vs. aroeira honey or wild honey).

was significant. The surface invaded by the cells of the control group was larger than the surface invaded by the Aroeira and wild honeys (Figure 5C).

DISCUSSION

Cancer of the oral cavity is the eleventh most common malignancy in the world (Petersen, 2005). OSCC is the most common type of oral câncer (Day et al., 2003). The development of new therapeutic agents aimed at the malignant behavior of these cancers is important to improve the prognosis of the treatment (Bramer et al., 2009). The study of the molecular mechanisms of chemotherapeutic agents and the combination of chemotherapeutic agents that induce synergistic antineoplastic activity are necessary to improve clinical outcomes (Kim et al., 2007). In this study, we focused on the investigation of the contributions of honey from the Jequitinhonha River Valley as antioneoplastic agent. This honey was chosen for the study since the antiproliferative and antimigratory effects of this type of honey have not yet been verified in any type of cancer cell line.

Studies have shown that honey may exert antineoplastic effects through several mechanisms (Eddy et al., 2008). The investigations indicated that honey has antineoplastic property through its interference with cell proliferation, apoptosis, cell cycle progression and mitochondrial membrane depolarization in various types of cancer, such as, skin cancer (Erejuwa et al., 2014), adenocarcinoma, cervical cancer (Pichichero et al., 2010), endometrial cancer (Tsiapara et al., 2009; Yaacob et al., 2013), liver cancer, colorectal cancer, prostate cancer (Davoodi et al., 2010; Samarghandian et al., 2011b; Samarghandian et al., 2014a), renal carcinoma (Samarghandian et al., 2014b), bladder cancer, lung cancer, osteosarcoma (Samarghandian et al., 2011a), leukemia and oral squamous cell carcinoma (OSCC) (Ghashm et al., 2010). In the present study, it was possible to verify that the antineoplastic action of honey is related to the modulation of cell proliferation and migration.

The bioinformatics analyzes conducted in the present study showed that the performance of honey in OSCC is dominated by the leader genes TNF, IL-6, and HDAC1. Histone deacetylases (HDACs) are key epigenetic regulators in gene expression and cell differentiation, proliferation, apoptosis, and inflammation (Secrist et al., 2003; Kato et al., 2009). In the OSCC microenvironment, both tumor cells and stromal cells release innumerable inflammatory cytokines, such as IL-6 or TNF, and increase the expression of their transcription factors: STAT3 for IL-6 and NF for TNF (Erdei et al., 2013; Feller et al., 2013). Some studies have shown that IL-6 induces angiogenesis and lymphangiogenesis, TEM (epithelial-mesenchymal transition) and resistance to chemotherapy (Shinriki et al., 2011; Yadav et al., 2011; Gao et al., 2016), while TNF increased *in vitro* the union between

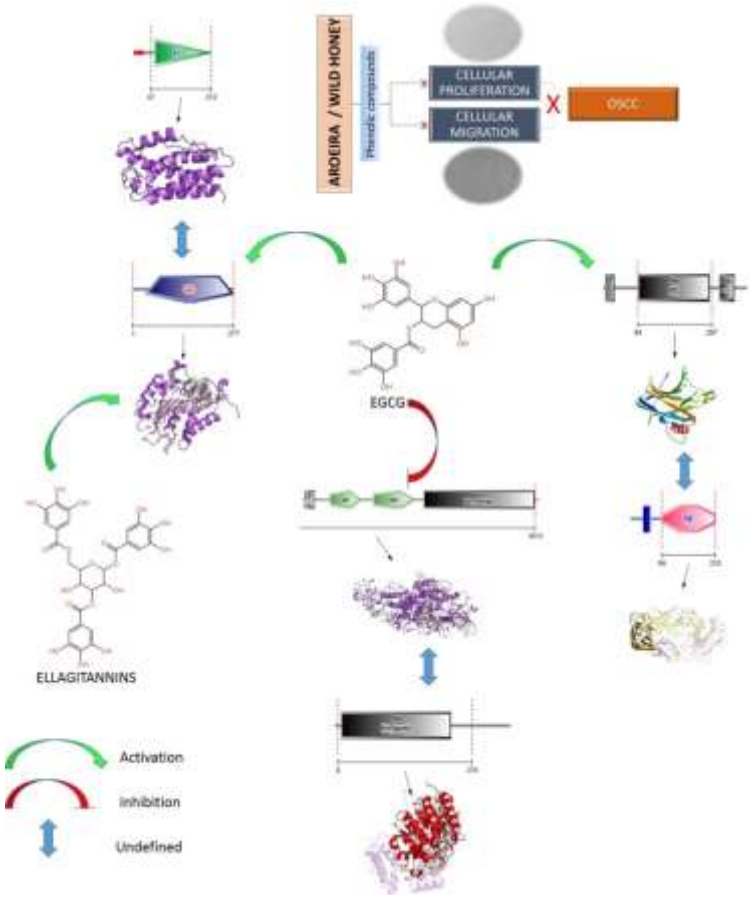

Figure 6. Performance of the honey from the Jequitinhonha River Valley - Brazil on the OSCC. A possible mechanism of antineoplastic action of honey through interference with cell proliferation and migration.

endothelial cells and OSCC cells, probably increasing the metastatic potential of this tumor (Song et al., 2012). TNF- is also related to the angiogenesis, growth and the tumor stem cell phenotype (Lee et al., 2012; Lai et al., 2016).

In an attempt to understand the molecular mechanism by which honey from the Jequitinhonha River Valley promotes antineoplastic action, the present study investigated the presence of bioactive compounds in honey to better elucidate these issues. Both in the honey of Aroeira and in the wild honey were identified important phenolic compounds as Steroids / Triterpenoids, Flavonoids Saponins, Alkaloids, and Tannins.

In general, honey contain a complex mixture of bioactive compounds, genetic inheritance of the botanical species that originated it, responsible for their activities such as aromatic aldehydes, aromatic carboxylic acids and their esters, derivatives of carotenoids, terpenoids, flavonoids, and others appear in minor proportions. Many of this wide range of minor constituents present in honey has antioxidant and antimicrobial properties, among them are phenolic compounds that also contribute to exalt their sensorial qualities (Gomez-Caravaca et al., 2006; Simon et al., 2009).

The action of polyphenols has been investigated in various cancers including lung, skin, oral cavity, ovarian, esophagus, stomach, liver, pancreas, endometrial, thyroid, testicular bladder, small intestine, colon, urinary tract, and prostate (Yang et al., 2002; Arts and Hollman, 2005). It has been demonstrated that polyphenols exhibit anti-cancer property by interfering with molecular events involved in the initiation, promotion, and progression stages (Lambert et al., 2005). Therefore, the present study leaves strong evidence that honey, through the action of polyphenols inserted in its composition, promotes the reduction of SCC-9 cells, reducing their proliferative and migratory capacity.

Bioinformatics analyzes showed that polyphenols can positively modulate Caspase and TP53 and negatively the DNMT1 gene. We hypothesized that the genes modulated by polyphenols are capable of interfering with the action of the leader genes favoring the proliferative and migratory behavior of neoplastic cells of the mouth (Figure 6).

Concerning the modulation of IL-6 or TNF by caspase, the amplification of the NF-κB-IL-6/TNF-α-STAT-3 signaling cascade occurs in most malignancies and facilitate the expression of pro-inflammatory and pro-survival genes (Hu et al., 2013), and it has been demonstrated that caspase-8 can orchestrate this pathway (Lamkanfi et al., 2007; Blander, 2014; Gurung et al., 2014). Another study demonstrated that the inhibition of caspase-8 decreased the levels of IKKα, which is closely associated with NF-κB activation, being thus speculated that caspase-8-dependent non-canonical inflammasome may represent the switch for tumor-associated inflammation (Terlizzi et al., 2015).

The association between TP53 and TNF has been reported in other studies. TP53 is mutated or defective in function, the TAp73 compensatory function for TP53 is repressed, possibly because the TNF-α promotes displacement of TAp73 from pro-apoptotic genes and reprograms its binding and activation of AP-1 sites (Si et al., 2016).

Finally, it is important to note that DNA methyltransferase 1 (DNMT1) maintains DNA methylation and is implicated in tumorigenesis (Chen et al., 2007; Dawson and Kouzarides, 2012; Yang et al., 2015). HDAC1 forms a complex with DNMT1 (Robertson et al., 2000), and that the protein stability of DNMT1 is regulated by posttranslational modifications of acetylation and ubiquitination (Du et al., 2010; Cheng et al., 2015). The binding of DNMT1 with ubiquitin specific peptidase 7 (USP7) is regulated by the acetylation of DNMT1 (Cheng et al., 2015).

Although the phytochemical analyzes performed in the present study are only qualitative, it is already indicative of the presence of important classes of chemical compounds in the honey from the Jequitinhonha River Valley, which deserve better investigation, to verify the association of these compounds with the therapeutic activities of honey produced in the region.

The present study showed that the Aroeira and wild honeys of the Jequitinhonha River Valley has dose-dependent antiproliferative and antimigratory potential in OSCC cell lines. And the honey of Aroeira showed even more than the wild honey its proliferative activity, that deserves to be investigated better.

Given this, the continuity and validation of this study can be based on the possible consequences of the economic, social and environmental impact that can lead to. In addition, it is an innovative study in the field of research because it is not considered in the consulted literature, studies addressing the potential of Aroeira, as an apiculture pasture in the Jequitinhonha River Valley, to increase the quality of honey, and especially promoting an adjuvant action of honey in therapy against OSCC.

Lastly, as the Jequitinhonha honey's sovereignty is demonstrated, beekeepers will be encouraged to join the activity, as an alternative form of income, social inclusion and the establishment of man in the field.

Conclusion

About the hypothesis that led us to the conduction of this study, it was possible to conclude that honey produced from the flowering of Aroeira and wild in the Jequitinhonha River Valley can reduce the proliferation and migration of OSCC cells, according to the diagram shown in Figure 6.

The honey produced from the flowering of Aroeira in the Jequitinhonha River Valley is informally recognized for its quality. So the analysis of the antineoplastic potential in OSCC cells was important, allowing the addition of value to the honey and open new markets.

It is always important to note that to maintain the characteristics and quality, the local flora should be maintained. The bioactive classes found in honey indicate a potential therapeutic effect of the product, which in the future can become a coadjuvant in the treatment of cancer, minimizing the toxic effects of conventional treatments. Bioactive compounds may act singly or synergistically and are linked to the product's geo-botanical characteristics, attributing unique characteristics to the honey analyzed. However, further investigations are needed to determine the molecular mechanisms involved in the antiproliferative and antimigratory potential of honey.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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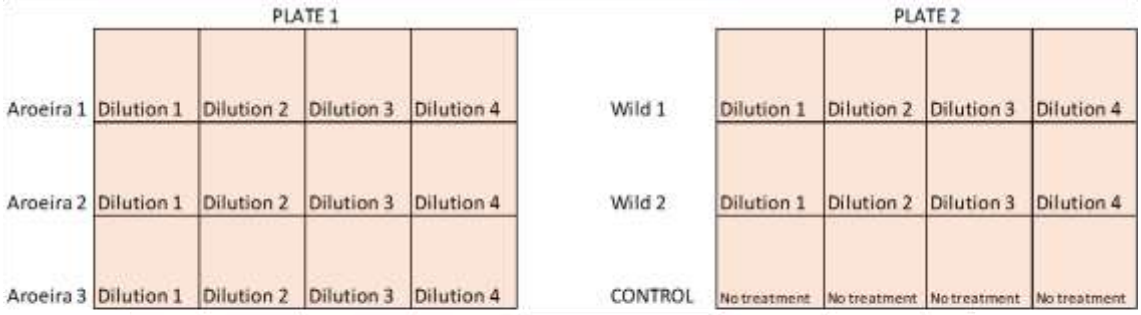
Supplementary Material



	Dilution 1	Dilution 2	Dilution 3	Dilution 4
Sample (µl)	500 of the pure sample	500 of the Dilution 1	500 of the Dilution 2	500 of the Dilution 3
Culture medium (µl)	1500	1500	1500	1500

Figure 1. Scheme of the experimental design of the treatment of cells with different samples of aroeira and wild honey. Scheme is representing a single experiment.

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