Research Article

In vitro cytotoxic effect of annona squamosa pulp extract as a mouthwash for children on human normal cell line

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Abstract: Background: Fruits and their by-products are the primary sources of bioactive chemicals in plants. Because of its phytochemical richness, Annona squamosa fruits have gained the alertness of people willing in health-promoting diets. The purpose of this in vitro study was to evaluate the cytocompatibility effect of ethanolic crude extract of Annona squamosa pulp against a human normal cell line as a mouthwash for children. Material and method: The ethanolic extract of Annona squamosa pulp was extracted using the ultrasonic method and then lyophilized to make it powder. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) test was performed to investigate the cytotoxic activity of the pulp extract on a human normal cell line derived from human dermal fibroblast, neonatal (HDFn). Plates were then incubated with 5% CO2 at 37°C For the following concentrations (400, 200, 100, 50, 25, 12.5, 6.25 ◎g/ml). Each concentration, as well as the positive control chlorhexidine, and the negative control cells without tested material, were tested in triplicate. Results: No significant difference was found between the cytotoxicity of the ethanol crude extract of Annona squamosa and a chlorhexidine (P = >0.05) against human dermal fibroblast of neonate cells, with IC50 (50% growth inhibition of cells) values of 235.4 µg/mL while chlorhexidine had an IC50 of 97.8 µg/mL. Conclusion: Annona squamosa extract is more safe and has less cytotoxicity than chlorhexidine. So, to overcome the problems of chlorhexidine, herbal mouthwash formulations could be utilized as an alternative mouthwash.

Keywords: Mosmann's Tetrazolium Toxicity assay (MTT assay), cytotoxicity, Annona squamosa, chlorhexidine, human dermal fibroblast of neonate (HDFn) cell line.

Introduction

The main etiological component contributing to periodontal diseases and dental caries is dental plaque (microbial biofilm). As a result, to avoid plaque production and accumulation on the tooth surface, effective plaque control techniques such as toothbrushes and dental floss, as well as mouthwashes as an adjunctive to these mechanical methods due to a lack of manual skill and motivation, particularly in children [(1)]. Chlorhexidine is regarded as the gold standard mouthwash and is the most widely recommended antibacterial and antiseptic agent. But unfortunately, tooth discoloration, burning sensations, oral ulcer and a change in taste after prolonged usage, all of which are reported side effects of chlorhexidine which limit patients' compliance and cause research about natural products to be safe with minimal adverse effects [(2)]. Despite the fact that the use of medicinal plants is an ancient tradition, it is still the major method for treating a lot of diseases in a diverse population and communities in many nations [(3)]. Herbal medicines are becoming more popular due to their fewer side effects on the biological environment and on non-targeted human cells [(4)]. As a result, plant extracts and their separated components have received a lot of attention in the study for newer medicinal agents [(5)]. According to the World Health Organization (WHO), traditional medicines are used by more than 80% population of the world to meet their needs for primary health care. Alkaloids, flavonoids, tannins, and phenolic compounds were thought to be the most important bioactive substances in plants [(6)]. Annona squamosa L. is a species of Annona (Annonaceae family) that is known by several names in various languages: sweetsop, sugar apple, sweet apple, and gishta [(7)]. It

is a small tropical tree with up to 8 meters height, with round or heart-shaped greenish-yellow fruit. The pulp is edible and beautifully aromatic, with a white-tinged yellow color. Every carpel has an oval, shining, smooth, and dark colored seed within [8]. Therefore, it is a popular plant that is mostly cultivated in gardens for its tasty fruit and high scenically appeal. All parts of this plant, including the leaves, barks, seeds, and fruits, possess medicinal chemicals and have thus been used to treat a variety of ailments because it contains a variety of phytochemicals such as alkaloids, acetogenins, flavonoids, saponins, phenols, sterols, carbohydrate and tannins [9].

The fruits are a high source of calcium, phosphorus and iron and are usually consumed fresh or used to produce sorbet or juice beverages. Flavonoids, alkaloids, and acetogenins are chemical compounds have all been derived from the seeds and other parts of these plants, therefore; antibacterial effects of flavonoids and alkaloids have led to their use in the treatment of medical conditions such as skin disease, intestinal worms, and eye inflammation [6,10]. The ethno botanical study on Annona squamosa's selective cytotoxic effect has increased its popularity and application in the treatment of numerous ailments [11]. Gubta et al. [12] showed by their study for one month on animals, that it is an edible fruit with great nutritional content and it shows protective activity at a specific dosage without any harmful effect on the heart and liver of rats. Antimicrobial activity refers to the process of destroying or inhibiting disease-causing bacteria. Several antimicrobial agents, such as antibacterial, antiviral, and antifungal substances, are employed for this purpose. For each of them, there are several modalities of action for infection suppression [13, 14].

The aerial portions of the plant have antibacterial, anti-diabetic, anti-hyperlipidemic, anti-microbial, antioxidant, anti-head lice effect, anti-tumour, hepatoprotective, insecticidal, anti-lipidperoxidative, mosquitocidal, anti-thyroidic [10]. The MTT assay can be used to test prospective medications in vitro and to investigate drug resistance in cell lines. It also aids in the determination of medication effects in vitro and clinical usage prognosis [15]. The test is the most sensitive, precise and widely used method for detecting cytotoxicity and cell viability following toxic chemical exposure [16, 17]. As a result, this assay is an extremely valuable tool for testing materials' cytotoxicity or discriminating between several cell lines because it generates considerable data in a short amount of time, has excellent sensitivity and reproducibility, and is affordable in cost [18, 19].

The present research focuses on the effect of Annona squamosa fruit pulp on cell growth viability after estimation of its antimicrobial effect as a mouthwash for children in another research. Organic extracts of Annona squamosa fruit pulp were tested on a normal cell line of human origin for antimicrobial effect, which could make it a source for generating innovative antimicrobial therapeutic products from nature.

Materials and Methods

Annona fruits collection

The Annona squamosa fruit (Lebanon) was collected from Baghdad's local market (Baghdad/Iraq). It was gently washed with distilled water to remove dust, then dried and peeled. The fruit pulp was cleared from the seeds and bark by hand, and the pulp was chopped into little pieces. After that, the samples were placed in a plastic container and shipped to a lab for extraction.

Alcoholic extraction of Annona fruit

The extract was prepared by the Ministry of Science and Technology in Baghdad, Iraq. The prepared fresh pulp was sonicated with ethanol at 60°C for 30 minutes. After that, the extract was evaporated by a rotary evaporator under a vacuum [20]. The alcoholic extract was kept in glass containers and frozen before being lyophilized to get powder extracts, Dimethyl sulfoxide (DMSO) was used to dissolve the extracted powder to prepare various dilutions at varying concentrations (400, 200, 100, 50, 25, 12.5, and 6.25) ◎g/ml according to a pilot study.

Cell line culture and maintenance

The human normal cell line from HDFn (human dermal fibroblasts of neonate) were cultured in Roswell Park Memorial Institute medium (RPMI 1640; Sigma USA). MTT assay was conducted in the Centre of Natural Product Research & Drug Discovery, University of Malaya, Malaysia. After the cells in the flask established a confluent monolayer, the cell sheet was rinsed in phosphate buffer saline after the growth medium was emptied. The cells were then treated with a 2 to 3 ml solution of trypsin. The cells were allowed to incubate for 1 minute at 37°C until they were dislodged from the container and became isolated cells instead of a single-celled layer. Then fresh, complete Rosewell Park Memorial Institute (RPMI) media was applied. Cells are then distributed at the desired concentration into culture flasks if required and maintained for 24 hours at 37°C in a 5 percent CO2 incubator [21].

Cell viability assay (MTT assay)

In vitro evaluation of the cytotoxicity of Annona squamosa extract by the most common, rapid, and simpler Mosmann's Tetrazolium Toxicity assay (MTT) uses a yellow dye (3-[4,5-dimethyllthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) that transforms into purple formazan. This reaction occurs by the splitting of the tetrazolium ring by the mitochondrial enzyme (succinate dehydrogenase) of living cells, and this enzyme is found only in healthy, viable cells [22].

In 96 plane base wells of micro-plates, normal cells (1x104 – 1x106 cells/ml) were grown in 200 ◎l as final volume per well of complete culture media. The plates were then incubated for 24 hours in a CO2 incubator at 37°C. The medium was withdrawn after the incubation period, and the wells were filled with the prepared concentrations of Annona squamosa extract and the positive control chlorhexidine 0.12% (6.25, 12.5, 25, 50, 100, 200, 400) µg/mL and incubated with 5% CO2 at 37°C for 24 hours. In the same plates, cells control without extract were included as a negative control. Every well-received 10 µl of MTT solution and then incubated for another 4 hours with 5% CO2. After that, the media were removed, and each well-received 100 µl of solubilization solution in order to dissolve crystals of formazan. The cytotoxic effect of the tested extract and the positive control were presented as an IC50 value, which means that (50% inhibitory concentration of normal cells). At a wavelength of 570 nm, the absorbance was measured using an ELISA reader [22]. Triplicates of every concentration, and even the positive controls, were employed. The effect was measured according to the equation:

Viability (%) = optical density of sample ×100% / Optical density control

Statistical analysis

Data description and analysis were performed using a one-way analysis of variance ANOVA to determine the level of significance at a p value of (0.05). Results were displayed as mean, standard deviation and statistical significances were executed by Graph Pad Prism version 8 (Graph Pad Software Inc., La Jolla, CA).

Results

The production of formazan crystals related to decrease MTT tetrazolium by living cells was measured to determine cell viability. The dose-response viability of human dermal fibroblast cells treated with the Annona squamosa extracts and the positive control chlorhexidine of different concentrations to determine the IC50 (50% growth inhibition of cells) by MTT assay are presented in Table 1 and 2 as the Data were expressed by means, standard deviation. Annona squamosa concentrations of 100, 50, 25, 12.5 and 6.25 caused less toxic effects to the cells. While higher cytotoxicity to the HDFn cells was observed as the Annona squamosa concentration increased. Moreover, the concentrations (50, 25, 12.5, and 6.15) ◎g/ml of both test and control have high cell viability than the concentrations (100,200,400) ◎g/ml, which means it is a dose-dependent cytotoxic effect of the substance. In addition, the IC value of this assay for Annona squamosa for all concentrations was higher than chlorhexidine (0.12%) (235.4 µg/ml and 97.8 µg/ml respectively) as shown in figure 1 (A and B).

Table 1: Cell viability of the Annona squamosa extract and the positive control chlorhexidine at different concentrations by MTT assay.

	Annona squamosa		Chlorhexidine	
Concentration	**Mean**	**Std. Deviation**	**Mean**	**Std. Deviation**
400	66.71	3.275	74.27	4.757
200	73.26	4.130	76.70	2.609
100	81.98	2.561	84.34	2.663
50	89.58	1.478	86.07	1.917
25	94.17	0.6581	95.22	0.8209
12.5	94.48	1.794	95.95	1.028
6.25	94.41	0.4818	95.95	0.2003

MTT= Mosmann's Tetrazolium Toxicity

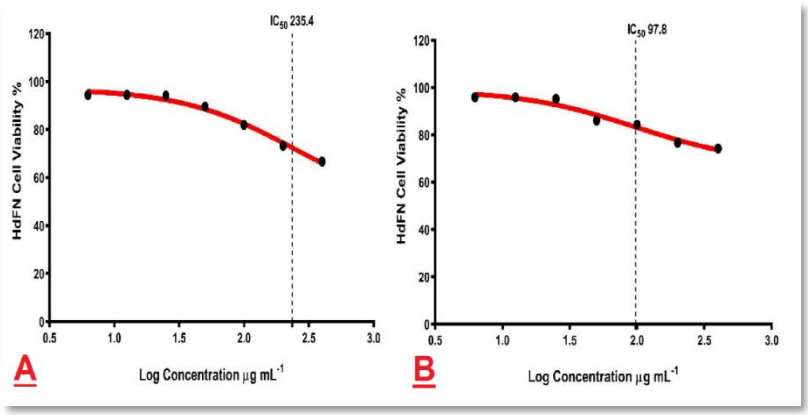

Figure 1: Half-maximal inhibitory concentration of cells on Human dermal fibroblast of neonate cell line of (A) Annona squamosa pulp extract (B) the positive control chlorhexidine gloconate 0.12%

Independent samples t-test was performed as demonstrated by table 3 and figure 2 to determine the significance of differences in cells viability mean values between the tested materials in different concentrations. The test showed no significant differences on HDFn cells between groups in all concentrations but only in concentration 400 ◎g/ml there was a significant difference between the Annona squamosa and the chlorhexidine.

Table 3: Independent samples t-test to compare the significance of differences in cells viability mean values between the Annona squamosa in comparison to Chlorhexidine gloconate 0.12%.

***A. squamosa* - Chlorhexidine gloconate**	**Adjusted P Value**	**Significance**
400	0.0048	S
200	0.4988	Ns
100	0.8598	Ns
50	0.4719	Ns
25	0.9984	Ns
12.5	0.9875	Ns
6.25	0.9832	Ns

NS= not significant at p>0.05, S=significant at p<0.05.

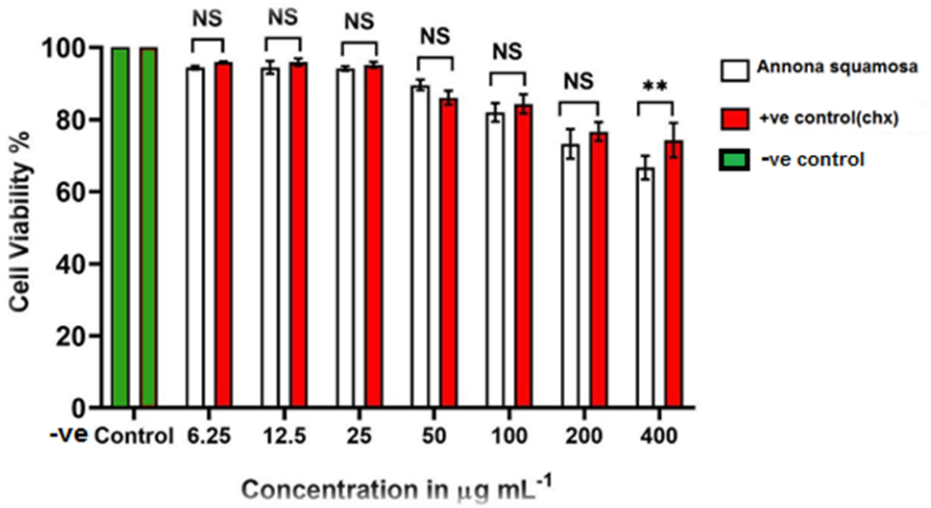

Figure 2: Graph showing the means of cells viability (%) of Annona squamosa and the positive control Chlorhexidine 0.12% and negative control at different concentrations.

Discussion

The MTT is the most commonly used test to identify the cytotoxic effect of various substances under various conditions or concentrations. Human dermal fibroblasts from neonates were applied in this study because their main properties were nearly equivalent to human gingival fibroblasts [23]. By comparing the survivability of the control and drug-treated groups, the IC50 value (50 percent half inhibitory concentration) of applicable medications may also be determined [24].

Consequently, this in vitro experiment was carried out to assess the cytotoxicity of *Annona squamosa* at various doses compared with chlorhexidine gloconate 0.12%. It was noted that the effect of *Annona squamosa* on HDFn after 24 hours was dose-dependent, meaning that low doses of *Annona squamosa* have minimal effect on cell viability, implying that exposure to *Annona squamosa* at the IC_{50} dosage resulted in considerable viability of human cells which is higher than that of chlorhexidine gloconate 0.12%, that's mean; halve of the concentration of viable cells are more when the *Annona squamosa* used (IC50 were 235.4 µg/ml and 97.8 µg/ml for *Annona squamosa* and chlorhexidine gloconate 0.12% respectively). The finding demonstrates that the higher the cell concentrations of *Annona squamosa*, the more toxic of the herbal agent above 400 µg/ml concentration, which is similar to the results obtained by another study regarding the safety and cytotoxicity of fruit's pulp in vivo and in vitro experiments [11].

Additionally, *Annona squamosa* was approved by FDA (Food and Drug Administration) [25] and it is safe fruit for eating. In regard to the comparison with chlorhexidine gloconate 0.12%, because this is a series of research that introduces the *Annona squamosa* pulp extract as a mouthwash for children which implicates the antibacterial effect of *Annona squamosa* compared with chlorhexidine, we search for this cytotoxic effect as compared with chlorhexidine, which is suitably used for children (if necessary) and considered the gold standard mouthwash at this concentration.

The use of medicinal plants in treating and preventing oral problems can have occasional advantages for rural populations or those in poor socioeconomic conditions due to the low cost and accessibility of

herbs in various portions of the country. As a result, medicinal herbs may be a viable substitute for traditional antimicrobials [26], as well as to avoid the side effects associated with chemical agents found in some medications used now.

Conclusion

The present study of ethanolic crude extract of *Annona squamosa* pulp and the positive control chlorhexidine gloconate 0.12% displayed different degrees of cell viability according to concentrations used, and the cell viability toward HDFn cells increased with decreased concentrations, so it is dose-dependent toxic effect. *Annona squamosa* has no or minimal cytotoxicity on human fibroblast cells and can be utilized in dentistry applications as a safe substitute to chlorhexidine gloconate 0.12% for prevention and treatment of oral bacterial-induced diseases.

Conflict of interest: None.

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العنوان: التأثير السام للخلايا المختبري لمستخلص فاكهة القشطة كغسول للفم للأطفال على خط الخلايا الطبيعي البشري

الباحثون: سمية حسين علي1 , زينب جمعة جعفر 2

المستخلص:

الخلفية: الفاكهة ومنتجاتها الثانوية هي المصادر الأساسية للمواد الكيميائية النشطة بيولوجيا في النباتات. بسبب ثرائها الكيميائي النباتي، اكتسبت ثمار انونا سكواموزا مؤخرًا انتباه الأشخاص الراغبين في اتباع أنظمة غذائية تعزز الصحة. كان الغرض من هذه الدراسة المختبرية هو تقييم تأثير التوافق الخلوي للمستخلص الخام الإيثانولي من لب أنونا سكواموزا ضد خط الخلية الطبيعي البشري كغسول فموي للأطفال.

المواد وطرق العمل: حضّر المستخلص الإيثانولي من لب انونا سكواموزا بطريقة الموجات فوق الصوتية ثم جمّد لعمل المسحوق. تم إجراء اختبار **MTT (3-(4 ، 5-** **2- (dimethylthiazol-2-yl)، 5-diphenyl-2H-tetrazolium bromide)** للتحقق من النشاط السام للخلايا لمستخلص اللب على خط الخلايا الطبيعي البشري المشتق من الخلايا الليفية الجلدية البشرية لحديثي الولادة **(HDFn).** تم تحضين الألواح بعد ذلك بـ 5٪ **CO2** في 37 درجة مئوية للتركيزات التالية (400، 200، 100، 50، 25، 12.5، 6.25 مجم / مل). وبعدها اختبار كل تركيز بالإضافة إلى العينات الموجبة والسالبة للمقارنة في ثلاث نسخ.

النتائج: لم يُعثر على فرق معنوي بين السمية الخلوية لمستخلصات انونا سكواموزا والكلورهيكسيدين **(P => 0.05).** أظهر مستخلص الإيثانول الخام من انونا سكواموزا القليل من السمية الخلوية ضد خلايا**HDFn** ، مع قيم **IC 235.4** ميكروغرام / مل بينما كان الكلورهيكسيدين يحتوي على **IC50** من 97.8 ميكروغرام / مل .

الاستنتاج: يُظهر مستخلص أنونا سكواموزا تأثيرًا طفيفًا على قابلية بقاء خط الخلايا الطبيعية للإنسان مع **IC50** أعلى من الكلورهيكسيدين. لذلك للتغلب على مشكلات الكلورهيكسيدين، يمكن استخدام تركيب غسول الفم من أنونا سكواموزا كغسول فم بديلو