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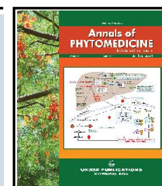

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Wound healing and cytotoxic effects of tannin rich fraction of *Psidium guajava* L. leaves

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Abstract

In India, the plant *Psidium guajava* L., also known as the guava, is widely distributed and a member of the Myrtaceae family. The whole plant is used in traditional medicine for various types of inflammation, wound conditions and commonly used as astringent, antiseptic and anthelmintic. Phytochemically, the plant was found to contain rich amount of tannin, a polyphenolic compound. Due to the presence of rich amount of tannin, the plant showed a remarkable wound healing property for any type of wounds. Therefore, the aim of the current study was to assess the cytotoxic and wound healing effects of the tannin-rich fraction using *in vitro* techniques. The presence of gallic acid in the isolated tannin rich fraction was confirmed by TLC analysis by comparing with standard. Cytotoxicity was evaluated using MTT method and *in vitro* cell proliferation and wound healing activity was determined by using scratch wound assay technique. The MTT assay result revealed no cytotoxicity because the proportion of cells that survived at the highest concentration (400 µg/ml) was above 80%. *In vitro* scratch wound assay in 3T3 mouse fibroblast cell showed that tannin rich fraction at 100 µg/ml had highest cell proliferation and migration 62.48% after 24 h exposure. As a result, the tannin-rich fraction of *P. guajava* leaves showed the ability to heal wounds by enhancing fibroblast proliferation and migration, and these results would support the traditional claims of *P. guajava* as a possible wound healing plant.

1. Introduction

A inevitable aspects of life are wounds. Different wound types are treated by various kinds of therapy. The human body has a natural self-healing system, which is a fortunate for human. Hemostasis, inflammation, proliferation, and remodeling are the four programmed stages that make up the normal biological process of wound healing in the human body. For healing to be successful, each of the four phases, soluble mediators, blood cells, extracellular matrix, and parenchyma cells - must take place in the right order and within a certain amount of time (Thakur *et al*., 2022). Chronic wounds and delayed acute wounds have typically not progressed through the normal stages of healing when they show signs of impaired healing. These wounds typically experience pathologic inflammation as a result of a delayed, imperfect, or disorganized healing process. The majority of chronic wounds are ulcers brought on by ischemia, diabetes, venous stasis disease, or pressure (Geethalakshmi *et al*., 2013). Herbal plants are more effective healers because they support the body's natural repair processes. It is crucial for the globe to have increased research on the alleged medicinal plants to come up with wound healing chemicals that are economical, effective, and safe because the bulk of currently available medications for wound treatment are expensive and pose issues like allergy and drug resistance (Mohd. Kashif Husain, 2021).

Numerous plants that contain polyphenols have antioxidant and antibacterial characteristics as well as the ability to promote the quick re-epithelialization of wounds (Naga Venkata Sai Lakshmi *et al.,* 2022). Among these medicinal plants, *P. guajava*, also known as guava, is a well-known traditional medicinal plant utilized in a variety of traditional medical systems. It is a member of the Myrtaceae family (Kaneria and Chanda, 2010). Pharmacological studies show that bark, fruit and leaves are used as antibacterial, hypoglycemic, anti-inflammatory, antipyretic, anticonvulsant (Begum *et al*., 2002). Traditional medicine uses the root extracts, bark, and leaves to treat wounds, ulcers, toothaches, coughs, sore throats, swollen gums, and several other inflammatory conditions (Dhara *et al*., 1994). *P. guajava* has been shown to contain tannins, triterpenes, flavonoids, and phenolic chemicals in phytochemical studies (Begumet *et al.,* 2002). Due to its high tannin content, it has been demonstrated to have antibacterial, analgesic, anti-inflammatory and wound healing effects (Sohafy *et al*., 2009; Jaya Kumari *et al*., 2018).

The aim of the current study was to assess the *in vitro* wound healing capacity of the tannin-rich portion of *P. guajava* in order to support the claim. The objective of the present study was to evaluate the cytotoxicity effect and wound healing activity of tannin rich fraction in 3T3 mouse fibroblast cell using scratch wound assay technique. Scratch assays performed in *in vitro* investigations, according to Fronza *et al.* (2009), offer the first insights into the

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beneficial role that plant preparations play in the development of new tissue. The findings of this study will provide insight of in-depth understanding of the correlation of tannins obtained from *P. guajava* leaves on the cell proliferation and migration of fibroblasts. Hence, it could be a promising platform for discovering natural remedies to enhance the wound healing process.

2. Materials and Methods

2.1 Materials

P. guajava leaves were procured for the intended study during the month of January from the Vels University campus garden in Pallavaram, Chennai. Dr. P. Jayaraman, Director of the Plant Anatomy Research Center (PARC), Tambaram and Chennai, recognized it and verified its authenticity. For future reference, a voucher specimen with the number PARC/2019/594 has been submitted.

2.2 Methods

2.2.1 Isolation of tannin rich fraction from *P. guajava* leaves

With petroleum ether, around 1 kg of coarsely powdered *P. guajava* leaves were defatted. Cold maceration was used to extract the defatted extract using acetone and water in a 70:30 ratio for 24 h with intermitten stirring. The fraction known as the tannin rich fraction was obtained after the solvent was filtered, distilled out, and the last remnants of the solvent were removed under vacuum (Mccallum *et al*., 1990).

2.2.2 Estimation of tannin content by UV spectroscopy method

Tannin content in the fraction was determined according to Ejikemeet *et al*. (2014). Folin-Denis reagent was prepared by dissolving 50 g of sodium tungstate (Na_2WO_4) in 37 ml of distilled water. 25 ml of orthophosphoric acid (H_3PO_4) and 10 g of phosphomolybdic acid ($H_3PMo_{12}O_{40}$) were added to the reagent that was previously made. The mixture was refluxed for two hour, cooled, and then diluted to a volume of 500 ml with distilled water. 100 ml of distilled water and one gram of each sample were combined in a conical flask. This was gently boiled for 1 h on an electric hot plate, then filtered using Whatman number 42 (125 mm) filter paper in a 100 ml volumetric flask. Following the pipetting of 5.0 ml of the Folin Denis reagent in a 100 ml conical flask, added 50 ml of distilled water, 10 ml of saturated Na_2CO_3 solution, and 10 ml of the diluted fraction (aliquot volume). After vigorous stirring, the solution was allowed to stand for 30 min in a water bath at a temperature of 25°C. The optical density was measured and the results were compared with tannic acid curve. Dissolution of 0.20 g of tannic acid in distilled water and dilution to 200 ml mark (1 mg/ml) were used to obtain tannic standard curve. Standard tannic acid solutions at various strengths (0.2–1.0 mg/ml) were pipetted into five distinct test tubes, to which the Folin Denis reagent, 5 ml of saturated Na_2CO_3, and 10 ml of distilled water were added. In a water bath set to 25°C, the solution was allowed to stand for 30 min.

A Spectrum Lab 23A spectrophotometer was used to measure optical density at 700 nm. Plotting was done between optical density (absorbance) and tannic acid content. The computation was performed using the following formula:

$$\text{Tannic acid (mg/100 mg)} = \frac{\text{C} \times \text{extract volume} \times 100}{\text{Aliquot volume} \times \text{weight of the sample}}$$

where, C is the tannic acid concentration as seen on the graph.

2.3 Thin layer chromatography analysis of tannin rich fraction

In 5 ml of water, 10 mg of the test sample's tannin-rich fraction were dissolved. A spot of one micro liter was administered. The reference sample was prepared by dissolving 10 mg of gallic acid in 5 ml of water. The mobile phase used was glacial acetic acid (10 ml) and detected by spraying with 5% ferric chloride as detecting agent (Gangwal, 2013). Following plate development, they were air dried, and the Rf value was computed. After spraying the detecting chemical, spots were seen by a UV chamber.

2.4 *In vitro* cytotoxicity studies

Cytotoxicity of tannin rich fraction was tested on vero cells. Various tannin-rich fraction concentrations (3.125-400 µg/ml) were produced in PBS and sterilized using syringe filters. Cell densities of 2×10^5 cells/ml were used for cytotoxic testing. Cells that had already been seeded in 96-well plates of the aforementioned density received additions of tannin-rich fraction (3.125-400 µg/ml) and were then incubated for 24 h. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the vitality of the cells. The culture was stained for 4 h at 37°C using MTT solution (5 mg/ml). After incubation, the medium was changed with 100 l of dimethyl sulfoxide (DMSO), and an optical microplate reader was used to measure absorbance at 570 nm (Beckmen coulter) (Jethva Khushboo *et al*., 2016).

2.5 *In vitro* wound healing activity

2.5.1 Preparation of cell suspension

After discarding the culture medium, 3T3 mouse fibroblast cells were subcultured in Dulbecco's Modified Eagle's medium (DMEM) and then trypsinized separately. In the flask, 25 ml of DMEM with 10% fetal bovine serum (FBS) were added to the dispersed cells. By gently passing the pipette through the medium while homogenizing the cells, the cells are suspended in the medium (Sara *et al*., 2017).

2.5.2 *In vitro* wound scratch assay

Using the scratch test technique, the migration rates of 3T3 murine fibroblast cells were evaluated. In a 24-well plate with a cell density of 2×10^5 cells, full media was added to each well before it was incubated at 37°C and 5% CO_2. The confluent monolayer of cells was horizontally scraped using a sterile 200 µl pipette tip after 24 h of incubation. Phosphate buffered saline (PBS) was used to wash the dirt away. By dilution with serum-free DMEM, the standard and test compounds were treated to the cells at a concentration of 100 µg/ml. The control cell was the one that received no treatment. Before incubating with the test and control substances, the scratch-induced wound was imaged at 0 h using phase contrast microscopy at a magnification of about 100 X. The second series of pictures was photographed 24 h after the first one. The percentage of the closed area was measured and compared to the figure obtained at 0 h to ascertain the migration rate. The percentage of the closed area increased, which denotes cell migration (Sara *et al*., 2017).

Wound closure (in %) = (measurement at 0 h – measurement at 24 h)/measurement at 0 h × 100

3. Results

The ethnobotanical studies revealed that the leaves of the plant *P. guajava*, are used for wound healing activity due to the rich content

of tannin. So, the present work was focused to isolate tannin rich fraction and evaluated for wound healing and cytotoxic effect by *in vitro* model.

The percentage yield of tannin rich fraction of *P. guajava* leaves was 25 % w/w. Estimation of tannin content in tannin fraction was done spectrometrically using Folin Denis reagent and the results showed that tannin fraction contains 9.74%w/w of tannin content. The presence of gallic acid in tannin rich fraction was identified by comparing with the standard sample gallic acid by TLC. The R_f value of standard (0.57) matches with the R_f value of test component (0.55) shown in Figure 1.

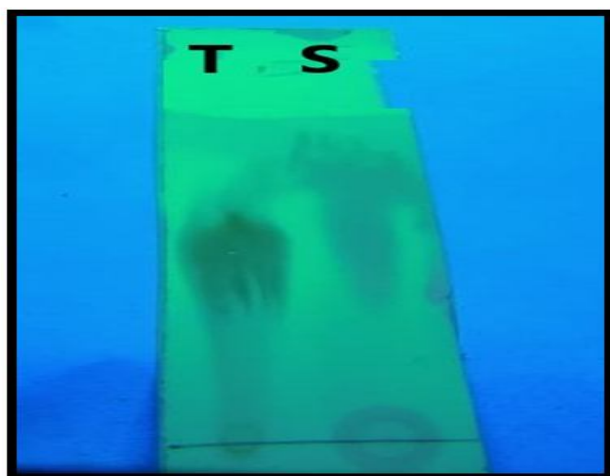

Figure 1: TLC chromatogram of tannin rich fraction (T) and standard (S).

3.1 *In vitro* cytotoxicity study

Cells were exposed to eight different concentrations, from 400 to 3.125 µg/ml, for tannin rich fraction. The results showed no cytotoxicity as cell survival percentage was above 80% at highest concentration (400 µg/ml) are shown in Table 1 and Figure 2. The outcome of the MTT colorimetric assay is the formation of purple-colored formazan crystals by active, live cells. Living cells' mitochondria transform MTT biologically into formazan. As dead cells cannot produce formazan, the formation of formazan has a direct relationship with live cells (Njeru and Moema, 2021). Over 80% of the cells were viable, demonstrating biocompatibility. Thus, tannin-rich fraction of leaves of *P. guajava* could be used as a secure chemical for application in the healing of wounds.

Table 1: *In vitro* cytotoxicity effect of tannin rich fraction of leaves of *P. guajava* and standard

Sample concentrations (µg/ml)	VERO cell viability (%)	
	S1A (Normal)	S1B (Pulse)
0	100.00	100.00
3.125	100.00	100.00
6.25	100.00	100.00
12.5	100.00	100.00
25	98.06	98.10
50	92.05	95.57
100	89.50	91.67
200	86.70	88.37
400	85.15	87.89

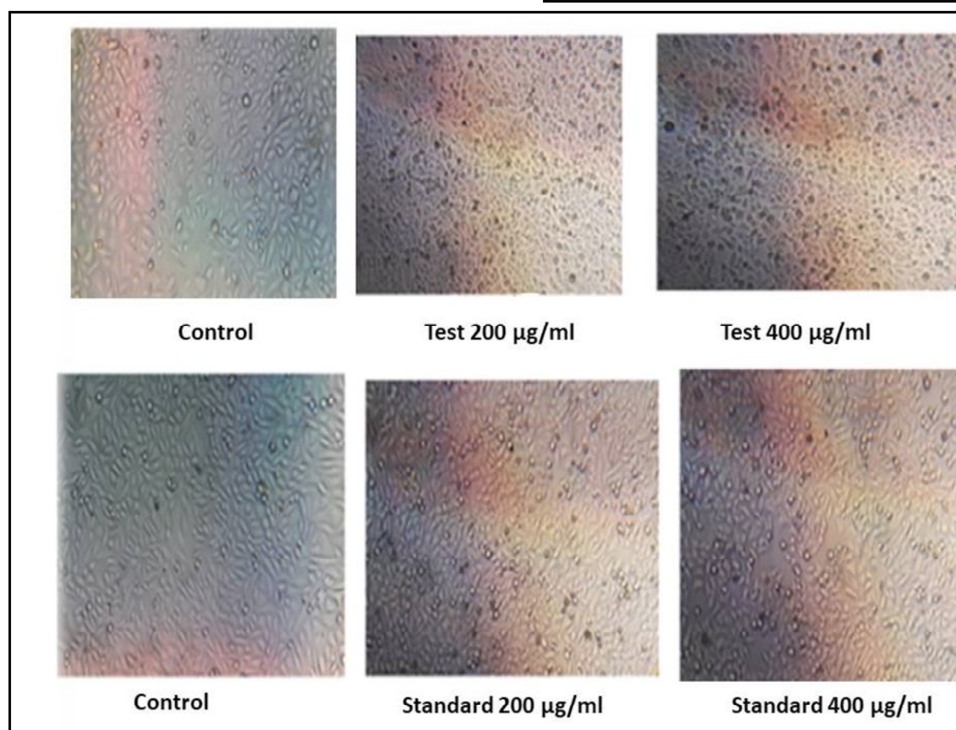

Figure 2: *In vitro* cytotoxicity of tannin rich fraction and standard on vero cell lines.

3.2 *In vitro* wound healing activity

On mouse fibroblasts, a tannin-rich fraction was tested to determine its capacity for wound healing. During a 24 h incubation period, the cellular proliferation and migration of fibroblasts were examined in the presence and absence of a tannin-rich fraction of leaves of *P. guajava*. After being exposed for 24 h to the test sample's tannin-rich fraction of leaves of *P. guajava*, it was found that, in contrast to the control, cell migration toward the provisional gap was induced.

The migration rate of cells tested for 24 h are shown in Figure 4. In comparison to untreated (control) cells, cell migration rates were increased by each of the test. In presence of tannin rich fraction of leaves of *P. guajava* and standard gallic acid, percentage cellular migration was found to be 62.48% and 70.01% when compared with control are shown in Figure 3. Tannin rich fraction of leaves of *P. guajava* treatment resulted in restoration of original cell number within 2 days when compared to control.

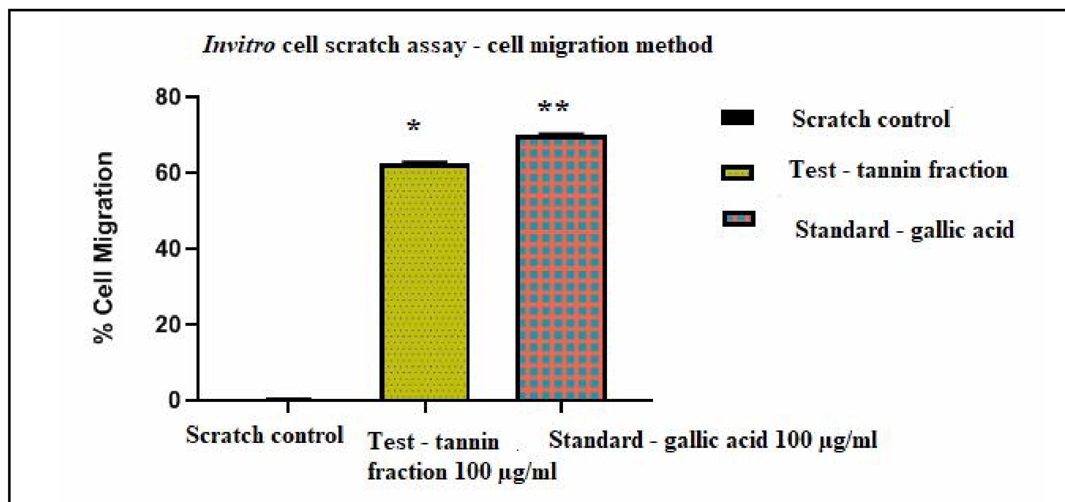

Figure 3: Percentage of cell migration of fibroblast cell treated with tannin rich fraction from *P. guajava* leaves and standard.

Data analysis for the cell migration method was performed by Graphpad prism version 8.0 One-way ANOVA was conducted to compare each group with the Scratch control group. *p* value shows *$p \leq 0.05$ to be significant ** $p \leq 0.01$ more significant.

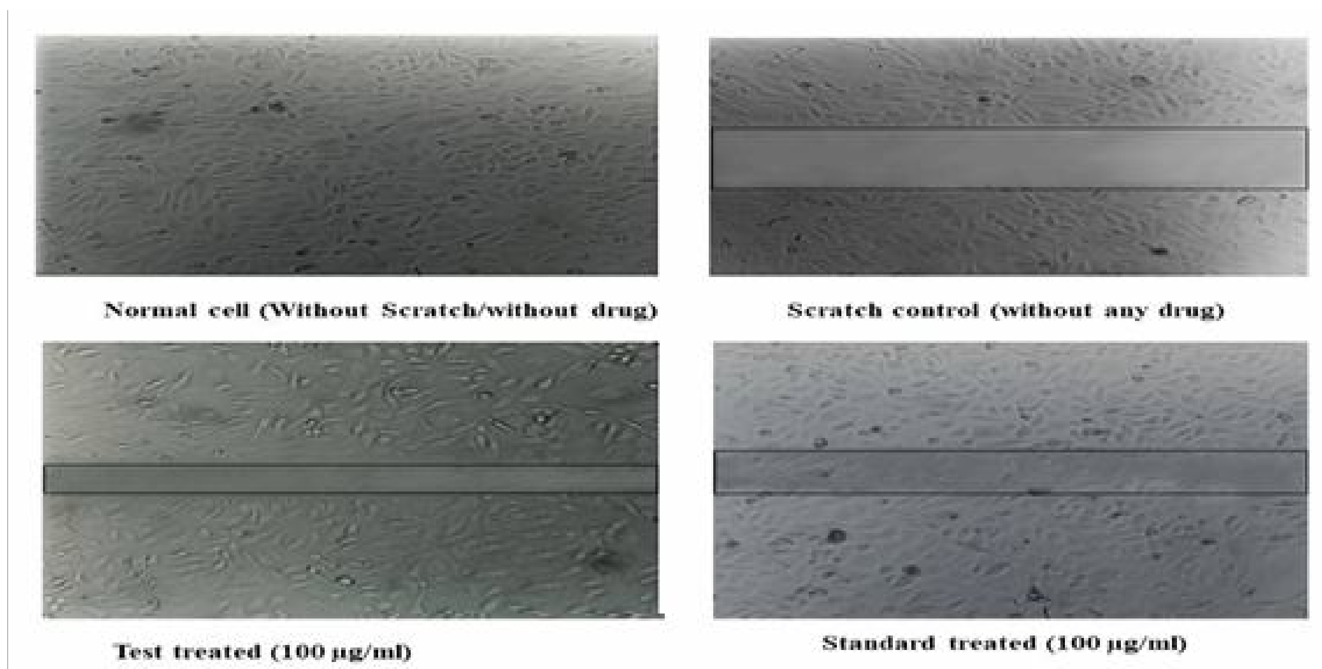

Figure 4: Cell migration of mouse fibroblast cells treated with test and standard in *in vitro* scratch model: Migration and proliferation of fibroblast cells were captured at (100 × magnification) using phase contrast microscope.

4. Discussion

A wide range of therapies were existing for healing wounds such as topical corticosteroids, antibiotics, low-power laser phototherapy, anti-inflammatory, immunosuppressive, and immunomodulatory drugs (Vena *et al*., 2006). However, repeated or prolonged use of corticosteroids suppresses the adrenal glands (Houghton *et al*., 2005). In the quest for drugs replacing corticosteroids for wound healing, the present study analysed wound healing property of tannin rich fraction from *P. guajava* leaves, which has been proven to maintain cell survival proved from the reduction of the terazolium salt by vero cells in a micro culture tetrazolium assay (MTT).

The *in vitro* cytotoxicity activity of tannin rich fraction was evaluated through micro culture tetrazolium assay (MTT) on vero cell line. This test is highly accurate, sensitive, and quantitative. It is predicated on the mitochondrial dehydrogenase enzyme's capacity to convert the yellow, water-soluble MTT into an insoluble, dark purple formazan that is formed in a variety of different cell lines in direct proportion to the number of cells (Ghasemi *et al*., 2021).

On mouse fibroblasts, the tannin fraction's potential for wound healing was assessed. The basic wound healing process involves proliferation, migration and functioning of fibroblasts and keratinocytes. Therefore, mouse fibroblast cell lines were selected as the best cell lines to employ for the *in vitro* study. Cellular migration is commonly studied *in vitro* using the scratch assay (Lee *et al*., 2012). Before new cellular contact is established, the cells on the edge of the freshly formed wound move toward the openings to close the "scratch". Under a phase contrast microscope, the scratch experiment was used to track the migration of mouse fibroblast cells in response to a fake injury. This method's ability to somewhat simulate *in vivo* cell movement is one of its main advantages (Liang *et al*., 2007).The term "rate of migration" describes how quickly cells move during a specific period of time. The cells often move in the direction of empty spaces. However, the environment has an impact on migration rates (Liang *et al*., 2007). Proliferation and migration of cells are crucial for wound healing, especially during re-epithelization, as the fast proliferated fibroblasts will provide a sufficient supply of cells to migrate rapidly and cover the wound site (Pastar *et al.,* 2024). The result of the present study showed that in presence of tannin rich fraction of leaves of *P. guajava* and standard gallic acid, percentage cellular migration was found to be 62.48% and 70.01% when compared with control. When compared to control, tannin rich fraction of leaves of *P. guajava* treatment led to the restoration of the original cell number within two days.

5. Conclusion

The goal of the current study was to investigate, using *in vitro* methods, the potential activity of the tannin-rich fraction from *P. guajava* leaves in wound healing. Within the experimentally tested concentration range (400-3.125 µg/ml), the tannin-rich fraction did not exhibit cytotoxicity. In wound healing study using *in vitro* scratch assay model, the tannin rich fractions contributed positively to fibroblasts proliferation and migration. Hence, the ethnical use of *P. guajava* for wounds and inflammations seems to be well supported by the abundant presence of polyphenolic compound tannin. The commercialization of traditional herbs may benefit greatly from studies on the efficacy of traditional herbs or plants. Therefore, it is believed that this research may help *P. guajava* to gain recognition as a tool for treating individuals with poor wound healing.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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