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Evaluation of the cytotoxicity of the aqueous extract of *Allium sativum* (Lilliaceae) on macrophage cells of the Raw-264.7 line by the cytotoxic concentration method (CC50)

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Abstract

The present work aims to identify, determine the presence and quantities of the chemical groups contained in the aqueous extract of *Allium sativum*, and to evaluate its cytotoxicity in order to justify its multiple biological activities and its therapeutic indications in traditional medicine for better evaluation in human health. An in vitro study was carried out at the bacteriology laboratory of the Central Hospital of Yaoundé and at the Nutrition and Nutritional Biochemistry laboratory of the University of Yaoundé 1. An aqueous extract of *Allium sativum* was prepared by cold maceration. To highlight the presence or absence of secondary metabolites, specific qualitative phytochemical tests based on discoloration, precipitation or turbidity reactions were carried out, using the methods of Pascual et al. [1] and [2]. The characterization of the chemical constituents was done by specific reactions. The investigation for these phytochemical groups were carried out on the aqueous extract following the protocols usually used the methods of Singleton et al. [3], Ghorai et al. [4] and Fernanda et al. [5]. The in vitro cytotoxicity of the extract was evaluated on Raw cells 264.7 murine macrophage cell lines, provided by the Noguchi Memorial Institute for Medical Research at the University of Ghana. The cells were maintained in continuous culture in the laboratory and subsequently used for the cytotoxicity test. The evaluation of the cytotoxicity of the aqueous extract of *Allium sativum* and the positive control (Dimethyl Sulfoxide) were carried out on macrophage cells of the Raw-264.7 line using the cytotoxic concentration method (CC50). The aqueous extract had a yield of 37.84%. The CC50 value for the aqueous extract of *Allium sativum* is greater than 1000 µg/mL, while for Dimethyl Sulfoxide (5%) the CC50 value is 0.08275 ± 0.00367 µg/mL. At the end of this study, we were able to evaluate the phytochemical screening of the plant. It appears that our extract contains high amounts of polyphenols, tannins, flavonoids and terpenoids. These results suggest an interesting potential of our extract with potential antimicrobial, anti-inflammatory, analgesic, vasculoprotective, antispasmodic, antioxidant and/or immunomodulatory properties. No significant cytotoxic effect of the aqueous extract of *Allium sativum* was observed on murine macrophages. These results suggest that the plant can be used in traditional therapeutic treatments. Furthermore, a more in-depth study by phytochemical sorting followed by chromatography will allow us to isolate the active molecules of *Allium sativum* in order to justify its multiple biological activities, its therapeutic indications in traditional medicine and in order to offer an available and affordable phytomedicine. accessible to the global community.

Keywords: Cytotoxicity; *Allium sativum*; Aqueous extract; Raw cells 264.7 murine macrophage cell lines; Cytotoxic concentration

1. Introduction

Immunity can be defined as ‘resistance to disease, and more specifically to infectious disease’, the immune system as ‘the set of cells, tissues and molecules that work together to resist infection’ and the immune response as ‘the

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coordinated reaction of these cells and molecules against pathogens'[6]. Although the main physiological function of the immune system is to prevent infections and eradicate overt infections, its impact extends beyond infectious diseases. Broadly speaking, the immune response involves two successive types of response: the innate immune response and the adaptive immune response. The innate immune response is the host's first line of defence against pathogens [7]. Present in all healthy individuals, it is effective immediately, at the point of entry of the microorganism. When the microbes manage to cross the epithelia, they are taken over in the underlying tissues by cells and soluble factors of innate immunity that contribute to their destruction. These soluble factors include pro-inflammatory cytokines, complement fractions and soluble innate immune receptors. The cellular players in innate immunity include myeloid cells such as phagocytes (macrophages (Mφ) and neutrophils (PNN)), as well as basophils, eosinophils, mast cells and lymphoid cells such as natural killer (NK) lymphocytes and other sub-populations of unconventional lymphocytes [8]. Depending on the nature of the aggression, the innate immune response generates two main types of response: an inflammatory response or an antiviral response. Mφs, polymorphonuclear cells and dendritic cells (DCs) express receptors that enable them to recognise microorganisms. Receptors for complement factors and immunoglobulins enable them to recognise opsonised microorganisms. Receptors known as PRRs (pattern recognition receptors) recognise molecular motifs expressed by large families of microorganisms known as PAMPs (Pathogen-Associated Molecular Patterns) [9,10]. Some of these PRRs also recognise motifs expressed by dead or damaged self cells DAMPs (Damage-Associated Molecular Patterns). Thus, Mφ and DC participate not only in the antimicrobial protective response but also in the control of tolerance to the self [6]. The term macrophages actually refers to a heterogeneous population of different cell subtypes with different and sometimes opposing functions. Macrophages therefore represent a wide range of cells which, depending on the tissue or the nature of the extracellular signals they receive, may polarise into macrophages specialising in the regulation of the immune response, tissue repair or inflammation. Macrophages play a central role in tissue homeostasis, host defence, inflammation and tissue repair [11].

They are also involved in tissue remodelling during ontogenesis and in the control of metabolic functions. Whereas Mφ and PNN are phagocytes specialising in the elimination of microorganisms, DC are specialized in the presentation of antigens. Compared with the innate immune response, the adaptive immune response appears later, is more specific to infectious agents and is the basis of immunological memory. The adaptive immune response is triggered/amplified by the presence of invasive microorganisms. M1 macrophages produce large quantities of IL-12 and little IL-10 (IL-12 high/IL-10 low), secrete pro-inflammatory cytokines such as IL-1 and IL-6, toxic intermediates of nitrogen (such as nitric oxide) and oxygen, and have strong microbicidal and tumoricidal activity [12]. There are different types of polarisation of human and murine M2 macrophages, initially described as regulators of M1 cell polarisation. The fundamental properties of macrophages are their mobility, phagocytosis associated with antigen presentation and secretory capacity [13]. This secretory capacity is multiple : complement factors, cytokines, haematopoietic factors, prostaglandins and free radicals. Macrophages play an important role in host defence against microorganisms, maintenance of tissue homeostasis (via clearance of senescent cells), metabolism of glucose, lipids, amino acids and iron, and remodelling and repair of damaged tissue [14]. M1 or M2 macrophages may be associated with a number of diseases (M1 : diabetes, atherosclerosis, allergy, rheumatoid arthritis, steatosis; M2: cancer, fibrosis, asthma, bacterial or viral infection). However, taking certain so-called toxic substances could have an influence on the course of the immune response and at the same time on the macrophages, which play a major role in the body's defence system. What's more, so-called modern treatments have harmful side-effects on this cell line [15]. As a result, the search for new molecules that are more active, cheaper and have fewer side-effects is now a matter of scientific urgency. Among these therapeutic alternatives, natural plants should be given pride of place. These natural plants offer a range of advantages by overcoming the problems associated with cost, accessibility and cultural barriers [16].

According to the World Health Organisation (WHO), 80% of African population depend on traditional medicine for primary health care [17]. It is known that medicinal plants are widely used in traditional medicine thanks to their antioxidant properties due to their phenolic compounds, anti-inflammatory, anti-allergic, vasodilatory [18] and free radical neutralising properties[19]. Unfortunately, these plants may contain highly toxic metabolites, even though they have powerful medicinal properties [18]. In this work, the scientific investigations focused on *Allium sativum* (Lilliaceae). This plant has the ability to synthesis secondary metabolites and is thought to be a major reservoir of chemically diverse compounds with a wide range of biological activities. As a reminder, several studies have examined the effectiveness of garlic as an antibacterial remedy. A 2012 study showed that garlic had antibacterial activity against several strains of bacteria, including Escherichia coli and Staphylococcus aureus [20].

Another 2013 study showed that garlic had antibacterial activity against Helicobacter pylori, a bacterium that can cause stomach ulcers and gastric cancer. In addition to its antibacterial properties, garlic also has antifungal and antiviral properties. It has been shown that garlic can inhibit the growth of several strains of fungi, including Candida albicans, and that it can also inhibit the replication of certain viruses, including the herpes simplex virus [21].

In addition, to our knowledge and in the light of the various research studies carried out, quantitative phytochemical screening and evaluation of the cytotoxicity of this plant on macrophages have not yet been carried out. The aim of this study is to strengthen the scientific basis for the use of this plant, in order to justify its multiple biological activities and therapeutic indications in traditional medicine. In this context, the research was devoted to a phytochemical study and an assessment of the cytotoxicity of the plant's aqueous extract on macrophages, which are key players in immunity processus.

2. Material and methods

2.1. Collection and processing of plant material

The *Allium sativum* extract was obtained from the garlic clove and not from the whole plant. *Allium sativum* cloves were obtained at the Mfoundi market in Yaoundé on 15 May 2024. They have been identified at the National Herbarium of Cameroon, thus obtaining Herbarium Nº44810/HNC. They were then taken to the Nutrition and Nutritional Biochemistry Laboratory, where they were cleaned and dried for four days in a ventilated oven at 65 °C until they reached a constant weight. They were then ground to a fine powder using a blender. The powder thus obtained was used to prepare the aqueous extract.

2.2. Aqueous extraction

A mass of 36 g of *Allium sativum* pod powder was weighed, then introduced into 220 mL of water and homogenized for 2 mins. The mixture was left to stand for 24 hours at room temperature. It was then filtered through cotton and Whatman No.2 paper respectively. The filtrate obtained was oven dried at 65 °C. The extract obtained was weighed and stored in a sterile bottle for further analysis. The garlic extract was inoculated onto Mueller Hinton medium and incubated at 37 °C for 24 hours to check sterility.

$$\text{Rendement} = \left(\frac{\text{Mass obtained}}{\text{Initial powder mass}}\right) \times 100$$

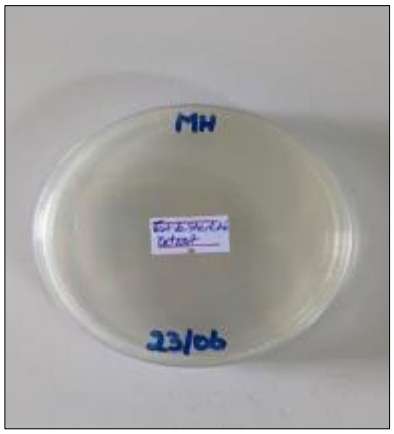

Figure 1 Sterility control of the aqueous extract *Al. sativum*

Figure 2 Dried *Allium sativum*

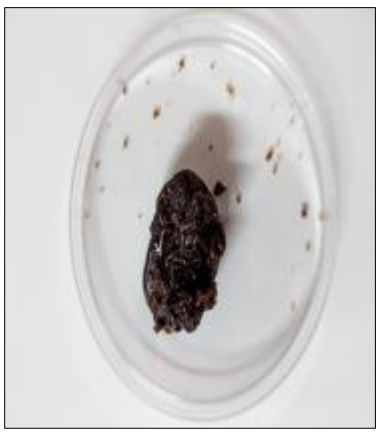

Figure 3 Aqueous extract of *Allium sativum*

2.3. Phytochemical Analysis

2.3.1. Qualitative phytochemical analysis

To identify the presence or absence of secondary metabolites, specific qualitative phytochemical tests based on decolourisation, precipitation or turbidity reactions were carried out, using the methods of [1, 2, 3, 22,] and completed by the methods of [23].

2.3.2. Quantitative phytochemical analysis

The quantitative phytochemical analysis of the stem barks of *Allium sativum* was determined by the standard methods.

Determination of Total Phenolic

Total phenolics were determined by using the method of [3]. In each test tube, we introduced 1000µL of distilled water, 100µL of 1 mg/mL extract and 200µL of Folin-ciocalteu (2N) solution. The mixture was left to stand for 3 min, then 2000µL of 20% sodium carbonate was added, followed by incubation in the dark for 1 hour at room temperature. The optical density was read at 750 nm. Calibration was carried out using gallic acid at concentrations of 200, 400, 600, 800 and 1000µg/mL. Total polyphenol levels were expressed in milligram equivalents of gallic acid per gram of extract (mg EqAG/g extract) using the calibration line with equation: y=0.0003x+0.0512, R^2 =0.9456.

Determination of total tannins

The test tubes containing volumes of 1.5 mL of acidic vanillin were added 500 µL of extracts (1 mg/mL). After homogenisation, the solutions obtained were incubated at room temperature for 15 minutes and the absorbance measured at 500 nm against the blank. Calibration was carried out using tannic acid at concentrations of 200, 400, 600, 800 and 1000µg/mL.

The total tannin content was expressed in milligram equivalents of tannic acid per gram of extract (mgEqAT/g extract) using the calibration line with equation y=0.0003x-0.0007, R2=0.9761.

Determination of Total Flavonoid Content

Total flavonoid were determined by using the method of Fernanda et al. (2015). The test tubes containing a volume of 1 mL extract (1 mg/mL) were added 1 mL AlCl3, 1 mL CH3COOK and 3.6 mL distilled water. After 30 minutes incubation at room temperature, the absorbance was read at 430 nm against the blank. Calibration was carried out using quercetin at concentrations of 200, 400, 600, 800 and 1000µg/mL. Flavonoid content was expressed as milligrams of Quercetin equivalent per gram of extract (mg EqQuer/g extract) using the calibration line equation y=0.0021x+0.0319, R^2=0.9856.

Quantification of terpenoids content

Terpenoids were quantified using the method of [4]. Two hundred microlitres (200 µL) of extract were added to 1.5 mL of chloroform. The mixture was homogenised by vortexing. Then 100 µL of concentrated 6N sulphuric acid was added to each tube. The mixture was cooled using an ice block for 10 min. Absorbance was measured at 538nm after 2 hours incubation at room temperature in the dark. Calibration was performed using oleanolic acid at concentrations of 200, 400, 600, 800 and 1000µg/mL. The calibration used is summarised in the table opposite: The terpenoid content was expressed as mg oleanolic acid equivalent per g extract (mg EqAO/g extract) using the calibration line with equation y=0.0005x+0.044, R^2=0.9358.

2.4. Cytotoxicity test

Cytotoxicity was assessed in a murine macrophage cell line by using the method of [24].

2.4.1. Murine macrophage cells

The in vitro cytotoxicity of the extract was assessed on Raw cells 264.7 murine macrophage cell lines, supplied by the Noguchi Memorial Institute for Medical Research at the University of Ghana. The cells were maintained in continuous culture in the laboratory and subsequently used for the cytotoxicity test.

2.4.2. In vitro culture of Raw cell lines

Raw cells were maintained in complete DMEM (1% antibiotic and 10% FBS) and stored in a 25 cm^3 culture flask (T-Flask) under standard conditions of 5% CO_2 at 37 °C. The culture medium was changed every 72 h. Once cell confluence (70-90%) was reached, the cells were detached by treatment with 1 mL of 0.05% trypsin-EDTA for 5 min. After inactivating the trypsin by adding 9 mL of complete DMEM, the detached cells were centrifuged at 1800 rpm for 5 min. The pellet obtained was then suspended in 1 mL of medium and cell viability was assessed using Trypan blue to calibrate the cell load on the Neubauer haemocytometer.

2.4.3. Determination of median cytotoxic concentrations (CC50)

As already mentioned, the cytotoxicity of the extract was assessed using the method described by [24]. The assay was performed in duplicate in sterile 96-well microplates. Briefly, 100 µL of cell suspension containing 1×10^4 cells was introduced into all wells of the plate and incubated for cell adhesion at 37 °C, 5% CO_2 for 18 h. Next, the cell culture medium was replaced with 90 µL of fresh medium, followed by the addition of 10 µL of test samples at different

concentrations (final extract concentrations: 1000, 500, 250, 125 and 62.5 µg/mL), in all wells except those that served as positive (DMSO, 20 µM) and negative (culture medium without test sample) controls. The plates were then incubated at 37 °C, 5% CO_2 for 48 h, after which 10 µL of a freshly prepared resazurin solution (0.15 mg/mL in PBS) was introduced into each well and incubated for 4 h at 37°C under 5% CO_2. Next, the fluorescence of the resulting preparation was measured using a microplate reader at excitation and emission wave lengths of 530 and 590 nm, respectively. From the resulting optical density values, the percentage cell viability was calculated with Microsoft Excel using the following equation :

$$\text{Percentage of cell inhibition} = \left(\frac{Ac - At}{Ac}\right) \times 100$$

At : Absorbance of test samples
Ac : Absorbance of negative control (Cells without extract)
A dose-response curve (cell viability versus sample concentration) was plotted using GraphPad Prism software to determine the median cytotoxic concentration (CC50).

2.5. Statistical Analysis

Values were presented as the mean ± SEM and data were analyzed statistically by one way analysis of variance (ANOVA) followed by Dunnett's test. P value less than 0.05 was considered significant.

3. Results

The results of the aqueous extraction of garlic (*Allium sativum*) are presented in the table below. The aqueous extract was obtained from a crude weight of 36g, giving an extract weight of 13.623 g, i.e. a yield of 37.84%.

Table 1 Extraction results

Plant species	Gross weight(g)	Extract weight(g)	Yield (%)
Allium sativum aqueous extract	36	13.623	37.84

3.1. Phytochemical Analysis

3.1.1. Qualitative phytochemical analysis

The phytochemical analysis of the aqueous extract of *Allium sartivum* revealed the presence of alkaloids, flavonoids, phenols, saponins, tannins and steroids (Table 2).

Table 2 Qualitative phytochemical of *Allium sartivum* aqueous extract

No	Constituents	Results
1	Alkaloids	+
2	Flavonoids	+
3	Saponins	+
4	Phenols	+
5	Steroids	+
6	Tannins	+

Key: (+) presence

3.1.2. Quantitative phytochemical analysis

Phytochemical analysis of the aqueous extract of *Allium sartivum* showed that it contains polyphenols, Tannins, flavonoids and Terpenoids. Total phenol content was 170.99±9.21 mg of gallique acid per gram of dried extract. Total

Tannins was 27.45±0.14 mg equivalent of tannique acid per gram of dried extract and Total flavonoids content was 16.16±1.34 mg equivalent of quercetin per gram of dried extract. Total Terpenoids content was 72.12±12.34 mg equivalent of oléanolique acid per gram of dried extract. (Table 3).

Table 3 Quantitative phytochemical *of Allium sartivum* aqueous extract

Allium sartivum	**Polyphenols (mg AG/g of dry extract)**	**Tannins (mg AT/g of dry extract)**	**Flavonoïds (mg QT/g of dry extract)**	**Terpenoids (mg AO/g of dry extract)**
Quantity	170.99±9.21	38.66±12.4	16.16±1.34	72.12±12.34

The results are expressed as mean ± SEM (n = 3). AG= gallique d'acid, QT= quercetin, AO= oléanolique acid, AT= tannique acid.

3.2. Results of the cytotoxicity test on Raw-264.7 macrophages

Table 4 Degree of toxicity of plant extracts by [25]

IC50 Toxicity	**IC50 Toxicity IC50 ≥ 0.1 mg/mL Non-toxic (-)**
	0.1 mg/mL > IC50 ≥ 0.050 mg/mL Low toxicity (+)
	0.050 mg/mL > IC50 ≥ 0.01mg/mL Medium toxicity (++)
	IC50 < 0.01mg/mL High toxicity (+++)

Cytotoxicity results are presented in the table below. The CC50 value for garlic extract is greater than 1000 µg/mL, while for DMSO the CC50 value is 0.08275 ± 0.00367 µg/mL.

Table 5 Cytotoxic concentration 50 of the aqueous extract

	Cytotoxic concentration 50 (CC_{50}) in µg/mL
Aqueous extract of *Allium sativum*	>1000
Positive control (DMSO)	0.08275 ± 0.00367

4. Discussion

This study tested the aqueous extract of *Allium sativum* and determined the cytotoxicity of the extract on a murine macrophage cell line Raw cells 264.7. Our results showed an extraction yield percentage of the aqueous extract approximately five times higher than those obtained by [26]. This discrepancy in the results could simply be explained by the fact that certain factors can influence the variation in extraction yield, including the plant species (variety), the organ used, the drying conditions of the plant, the metabolite content and the nature of the solvent used by [27]. This study showed that the aqueous extract was non-toxic, after comparison with the Mousseux scale. The phytochemical screening showed that the aqueous extract of *Alluim savitum* contained polyphenols, tannins, flavonoids and terpenoids. The presence of these chemical groups is in agreement with the work of [28]. Polyphenols are a large group of natural substances widely distributed in the plant kingdom, of which flavonoids are the main group. These substances have colouring, aromatic, medicinal and cosmetological properties that give plants adaptive advantages. In fact, they play a very important role in the plants that produce them. These molecules contribute to plant growth and the fight against pathogens [29].

The many studies that have shown the positive impact of polyphenol consumption on health and disease prevention mean that manufacturers are now marketing polyphenol-enriched foods and food supplements. In addition, their antioxidant activity ensures better preservation of foodstuffs by preventing lipid peroxidation. In the cosmetics industry, phenolic compounds find their practical application in combating the production of free radicals that are harmful to the health and beauty of the skin. In phytotherapy, phenolic compounds are used for their vasculoprotective, antispasmodic, anti-inflammatory, antioxidant and antimicrobial properties [2]. Saponins are a class of molecules characterised by their surface-active properties, as they dissolve in water to form foaming solutions [30]. They also have

anti-inflammatory, antifungal, antimicrobial, antiparasitic, cytotoxic, antitumour, immunostimulant and immunomodulatory properties [31].

This would mean that the aqueous extract is not toxic to human erythrocytes. However, the fact that a plant drug is not toxic does not mean that the recipe in which it is used is either. Pharmacological tests of the various recipes involving these plants are always of interest. Under these conditions, only recipes using a single non-toxic drug can be considered free of toxicity.

Evaluation of the cytotoxicity of the aqueous extract of *Allium sativum* and the positive control (DMSO) was carried out on macrophage cells of the Raw-264.7 line using the CC50 method. The CC50 result for the *Allium sativum* aqueous extract was greater than 1000 µg/mL, while for DMSO the CC50 value was 0.08275 ± 0.00367 µg/mL. These quantitative data indicate that *Allium sativum* aqueous extract exhibits low cytotoxicity on Raw-264.7 macrophage cells compared with DMSO, which is considered a reference compound with high cytotoxicity.

The results obtained clearly demonstrate that the aqueous extract of *Allium sativum* has excellent biocompatibility and very low toxicity on RAW-264.7 macrophage cells, which is in agreement with the work of [32]. Plant extracts causing 50% or more cell death at 1000 µg/ml are considered cytotoxic [33]. Cytotoxic tests performed on DMSO-treated cells showed a progressive decrease in violet staining in each well. As the dye only penetrates living cells, the staining was weaker the more cytotoxic the compound was by inhibiting DMSO-treated cells [34]. The sharp drop in the relative number of living cells treated with DMSO could be explained by the fact that this compound is highly toxic. This product contains a chemical compound that inactivates succinate dehydrogenase, an enzyme important for mitochondrial respiration, the blockage of which leads to cell death. The results obtained clearly demonstrate that garlic extract has excellent biocompatibility and very low toxicity on RAW-264.7 macrophage cells, which is in line with the work of [32]. Unlike DMSO, which is highly cytotoxic, garlic extract showed no significant adverse effect on cell viability, even at very high concentrations.

These data are particularly important in the context of developing new therapeutic approaches, as they suggest that the aqueous extract of *Allium sativum* could be used as a safe and well-tolerated bioactive agent. Given that macrophages play an essential role in the immune response, these positive results pave the way for future investigations into the potential antimicrobial, anti-inflammatory, analgesic, vasculoprotective, antispasmodic, antioxidant and/or immunomodulatory properties of the plant extract. On the basis of the results obtained, we can confirm that the recipe derived from the decoction of this plant is non-toxic. Subsequent studies can evaluate their preservation, in order to offer the community a low-cost phytomedicine.

5. Conclusion

At the end of this study, we were able to evaluate the phytochemical screening of the plant. Our extract was found to contain high levels of polyphenols, tannins, flavonoids and terpenoids. No significant cytotoxic effect of the aqueous extract of *Allium sativum* was observed on murine macrophages. These results suggest an interesting potential of our extract with potential antimicrobial, anti-inflammatory, analgesic, vasculoprotective, antispasmodic, antioxidant and/or immunomodulatory properties. In addition, further study using phytochemical sorting followed by chromatography will enable us to isolate the active molecules of Allium sativum in order to justify its multiple biological activities and therapeutic indications in traditional medicine.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest

Statement of ethical approval

As per international standard or university standard written ethical approval has been collected and preserved by the authors.

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