Antimicrobial potential of Pakistani medicinal plants against multi–drug resistance *Staphylococcus aureus*

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**ABSTRACT**

**Objective:** To determine resistance patterns of *Staphylococcus aureus* (*S. aureus*) isolated from different areas of Pakistan and to identify antimicrobial agents against multi–drug resistant *S. aureus* strains.

**Methods:** A total of 67 samples (sewerage, nasal and milk) were collected from different farm areas of Pakistan to identify local strains of *S. aureus*. Sixteen out of 67 samples were positive for *S. aureus*. Only 6 out of 16 *S. aureus* strains showed resistance to antibiotics. Then the antibacterial effect of 29 medicinal plants was evaluated on these *S. aureus* isolates and a standard *S. aureus* strain ATCC 25923. The solvents used for the extraction of plants were acetone, dimethyl sulfoxide and methanol. The in vitro antibacterial activity was performed using agar disc diffusion method. Moreover, minimum inhibitory concentration of effective medicinal plant extracts was identified through micro–dilution method to find out their 50% inhibitory concentration.

**Results:** Plant extracts of 5 medicinal plants (*Psidium guajava*, *Nigella sativa*, *Piper nigrum*, *Valeriana jatamansi*, and *Cucurbita pepo*) exhibited antibacterial activity against locally isolated multidrug resistant strains of *S. aureus*. The minimum inhibitory concentration of these extracts was ranged from 0.328 to 5.000 mg/mL.

**Conclusions:** Plant extracts of *Psidium guajava*, *Piper nigrum* seed, *Valeriana jatamansi*, *Cucurbita pepo* and *Nigella sativa* showed significant in vitro antibacterial activity and thus, such findings may serve as valuable contribution in the treatment of infection and may contribute to the development of potential antimicrobial agents against multi drug resistant strains of *S. aureus*.

**KEYWORDS**

*S. aureus*, MRSA, Antimicrobial agents

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**1. Introduction**

*Staphylococcus aureus* (*S. aureus*) is a Gram–positive, non–motile, non–spore forming, facultative anaerobe, catalase and coagulase positive bacteria with diameter of 0.5–1.5 µm and belongs to *Staphylococcaceae* family[1]. *S. aureus* is considered to be a major pathogen of both humans and animals and is causative agent of contagious bovine mastitis, a serious disease of livestock resulting in both reduced milk production and quality. *S. aureus* is found naturally on the skin, mucous membranes and in the nasopharynx of the human body. Some members are normal micro–flora of skin and mucous membranes; while other causes skin pustules, impetigo, suppuration, abscesses formation, septicemia, pneumonia, osteomyelitis, renal abscess, endocarditis, meningitis, gastroenteritis and toxic shock syndrome. Pathogenic *S. aureus* produces variety of extracellular enzymes and heat stable enterotoxins which
cause food poisoning[2].

Multi-drug resistance in *S. aureus* strains is a serious cause of concern in dairy animals and humans. The four main mechanisms by which microorganisms exhibit resistance to antimicrobials are drug inactivation, alteration of target site, alteration of metabolic pathway and reduced drug accumulation[3]. Strains of *S. aureus* that are resistant to β-lactam antibiotics are known as multidrug resistant *S. aureus*. Penicillin resistant strains of *S. aureus* have been observed to be multi-drug resistant against aminoglycosides, macrolides, quinolones and tetracycline, which are often used in the treatment of mastitis[4]. Penicillin resistant strains of *S. aureus* typically produce an enzyme, called a β-lactamase, which inactivated the β-lactam and deactivate penicillin. It becomes difficult to successfully treat mastitis because drugs are not able to penetrate to all infection sites and because the bacteria can avoid contact with antibiotics while residing inside leukocytes[5].

The development of antibiotic resistance is a multifactorial. To overcome the problem of antibiotic resistance, medicinal plants have been extensively studied as alternative treatments for different viral and bacterial diseases[6-11]. The current study made an attempt to find out novel anti-bacterial agents against multidrug resistant *S. aureus*. Penicillin resistant strains of *S. aureus* from medicinal plant extracts. For this purpose, twenty nine Pakistani medicinal plants were collected and screened for their antibacterial activity against multi-drug resistant *S. aureus*. Moreover, minimum inhibitory concentrations (MIC) of these plant extracts were identified through micro-dilution method to find out 50% inhibitory concentration of extract. Hopefully the resulting herbal extracts with antibacterial activity will be helpful for future to study these extracts in detail, and also to find out new therapeutic agents for better management of infections with multi-drug resistance *S. aureus*.

### 2. Materials and methods

#### 2.1. Sample collection

Different samples (milk, sewerage, and nasal) were collected for isolation of *S. aureus*. A total of 66 samples were collected, out of which 11 buffalo nasal samples were collected from University of Veterinary and Animal Science (UVAS), Lahore, 25 sewerage samples were from different areas of Lahore, and 30 milk samples of cows and buffalo were collected from different farms of Punjab. Milk and sewerage samples were stored at -20 °C, while nasal swabs were kept in normal saline at 4 °C.

#### 2.2. Plant collection and extraction

The plant materials from 29 plants were collected from different areas of Pakistan on the basis of un-documented reports (Table 1). The plants were identified by Pakistani plant taxonomist. The fresh plants were dried under shade at room temperature (20−25 °C) for 7 d. The dried plants were soaked in different solvents and extracts were filtered on the following morning. Extracts of each plant were concentrated at temperature between 30 to 40 °C and were evaporated to dry and then stored in refrigerator. About 20 mg of each dried plant extract was dissolved in 500 µL of respective solvent to make the final concentration of 40 mg/mL.

#### 2.3. Antimicrobial susceptibility activity

The antimicrobial susceptibility testing was performed using Kirby–Bauer modified disc diffusion method[12]. At least three to five well-isolated colonies were selected from an agar plate and transferred into a tube containing 4 to 5 mL of trypic soy broth. The broth culture was incubated at 37 °C for overnight in shaker. To achieve the turbidity equals to 0.5 McFarland standards, 50 µL of overnight culture was added in 5 mL of trypic soy broth and incubated at 37 °C for 1.5 h. Spectrophotometer was used to measure the optical density. In case of *S. aureus* the optical density should between 0.3−0.6. Muller–Hilton agar plates were prepared for disc assay. The disc diffusion technique and zone interpretation of each antimicrobial agent was used in accordance with National Committee for Clinical Laboratory Standards (NCCLS) guidelines[13]. The ampicillin (10 μg), tetracycline (30 μg), kanamycin (10 μg), erythromycin (15 μg) and oxacillin (1 μg) were used for drug susceptibility testing. The plates were then incubated at 37 °C for 19 h and the results were interpreted according to NCCLS standards for antibiotics susceptibility testing.

#### 2.4. Antimicrobial analysis of plants extracts

The antimicrobial activity was determined on multi-drug resistant *S. aureus*. Standardized inoculums were prepared. Sterile filter paper discs previously soaked in a known concentration of extract (40 mg/mL) was carefully placed at the center of the labeled plate. The plates were incubated aerobically at 37 °C in incubator and examined for zones of inhibition after 19 h.

#### 2.5. Determination of MIC

MIC is the minimum concentration that can inhibit the growth of a microorganism after overnight incubation. The
Table 1

Plants selected for antibacterial activity against multidrug resistant *S. aureus*.

| Species Family | Vernacular names | Part used | Local uses |
|----------------|------------------|-----------|------------|
| *Cinnamomum zeylanicum* (DARM) | Cinnamon | Bark | Analgesic, antibacterial, antifungal, antioxidants, anti-inflammatory, anti-viral, aphrodisiac, aromatic |
| *Acacia nilotica* (AN) | Mimosaceae | Indian Gum | Root | Used for the treatment of stomatitis, wounds, hemorrhage, ulcers, dysentery, diarrhea, skin diseases, burning sensation, cough, and dental caries |
| *Santalum album* (SAM) | Santalaceae | Indian Sandalwood | Flower | Useful in skin diseases |
| *Azadiracta indica* (AZD) | Meliaceae | Neem | Leaf | Anthelmintic, antifungal, antidiabetic, antibacterial, antiviral, contraceptive, sedative, blood purifying properties |
| *Taraxacum officinale* (TolI) | Asteraceae | Dandelion | Leaf | Used for gallbladder, diabetics, Loss of appetite and act as mosquito repellent |
| *Diospyros peregrine* (DPSM) | Ebenaceae | Panicha | Seed | Chronic dysentery, menorrhagia and excessive salivation |
| *Artemisia absinthium* linn (AFM) | Asteraceae | Common Wormwood | Leaf | Tonic, stomachic, febrifuge, anthelmintic |
| *Berberis lycem* (ZSM) | Berberidaceae | Ishkeen Kushnum Zarch | Flower | Used in the treatment of eye complaints, menorrhagia, chronic diarrohea and piles |
| *Tamarindus indica* (IND) | Fabaceae | Ambli | Flower | Cathartic, astringent, febrifuge, antiseptic, refrigator |
| *Nymphaea alba* (N.ALB) | Nymphaeaceae | White Lotus | Leaves | It acts as an astrigent antiseptic, anesthetic, aphrodisiac and sedative |
| *Fagonia cretica* (FCWM) | Aizoaceae | Dhamsha | Whole plant | It has medicinal potential, especially against cancer and tumors |
| *Nardostachys jatamansi* (NJRMD) | Valerianaceae | Sambul latebd Balchar | Root | Effective against kidney stones, jaundice, removes blood impurities, nervous headache, convulsions, respiratory and digestive diseases, skin conditions, typhoid, gastric disorders |
| *Cucurbita pepo* (CPLM) | Cucurbitaceae | Pumpkin | Leaves | It is used in treating bladder disorders, stomach upsets, intestinal worms, bed-wetting, rheumatism, benign prostatic hyperplasia, burns, and wounds |
| *Piper nigrum* (PNSM) | Piperaceae | Black pepper Kali mirch | Seed | It possesses anti-tumorigenic, immunostimulatory, stomachic, carminative, antihypercholesterolemic and again known for its strong phytochemical activities |
| *Trianthema portulacastrum* (TPW) | Aizoaceae | Bishkapra | Whole plant | Analgesic, purgative, stomachic, used for the treatment of anemia, bronchitis, piles, inflammation, liver troubles, asthma, itch, chronic ulcer, night-blinding, diseases of blood and skin |
| *Syzygium cumini* (SCLM) | Myrtaceae | Jamun | Leaves | Plant pacifies diarrhea, diabetes, leucorrhoea, fever, skin diseases and general debility |
| *Vitis vinifera* (VVLM) | Vitaceae | Grape seed | Leaves | It helps in burning sensation, constipation, hemorrhoids, anemia, skin diseases, colic, flatulence, jaundice, vomiting, splenomegaly, arthritis, and amenorrhea |
| *Musa paradisical* (MPTM) | Musaceae | Banana | Leaves | It provides large amounts of vitamins B-6 and C |
| *Moringa oleifera* (MOLM) (MOPM) | Moringaceae | Sahajna Pods | Anti-inflammatory, cancer treatment, gut treatment, Alzheimer prevention, relief for gastric ulcers, control of herpes symptoms, high cholesterol prevention, powerful natural antibiotics, HIV and AIDS treatment |
| *Valeriana jatamansi* (VJRM) | Valerianaceae | Samayo Sugandhawal | Root | Used as a sleeping and calming agent, used for sedative, mild anodyne, hypnotic, spasmyloytic, carminative, hypotensive; Indicated for hysterical states, excitability, insomnia, hypochondriasis, migraine, cramp and rheumatic pain |
| *Citrus sinensis* (SLM) | Rutaceae | Orange peel | Leaves | It strengthens the immune system, aid digestion and lowers the risk of food-borne pathogens |
| *Chenopodium album* (CAWM) | Chenopodiaceae | Fat hen | Whole plant | Acts as anthelmintic, antipathologic, antirheumatic, mildly laxative, odontalgic |
| *Terminalia arjuna* (TAFM) (TAFD) | Combretaceae | Arjuna | Flower | Analgesic and anti-inflammatory properties. Cardiomyopathy like myocardial infarction, angina, coronary artery disease, heart failure, hypercholesterolemia, hypertension. |
| *Panico granatum* (POM) | Lythraceae | Anaa | Fruit coat | It has immunostimulatory, anti-oxidant, anti-inflammatory anti-diabetic and anticaner |
| *Nigella sativa* (KAL) | Ranunculaceae | Kalonji | Seed | Use to relieve the symptoms of asthma, bronchitis, and coughing, to treat abscesses and tumors of the abdomen, eyes, and liver |
| *Pavidium guajava* (GUA) | Myrtaceae | Anmrood | Leaves | Use against cancer, bacterial infections, inflammation and pain |
| *Momordica charantia* (BGM) | Cucurbitaceae | Karela | Leaves | Act as antihelmitic, antimalarial, antiviral, anticancer and cardioprotective |
| *Avicennia marina* (AMLC) | Avicenniaceae | Mangrove | Leaves | Used for the treatment of ulcers |
| *Grewia asiatica* (GALM) | Malvaceae | Falsa | Leaves | The leaves are used as an application to pustular eruptions |
lower the MIC, the better is the antimicrobial agent. MIC was determined by micro–dilution method using serially diluted (10 folds) plant extracts according to the NCCLS 2000[13].

2.6. Statistical method

All statistical analysis was done using SPSS software (version 11.0, SPSS Inc). Data is presented as mean ± SE.

3. Results

3.1. Antimicrobial susceptibility

Disc diffusion assay was performed on 17 isolates of S. aureus. Due to the relatively small size, no separate analysis was undertaken for sewerage, milk and nasal isolates of S. aureus. In this study S. aureus were found to be highly resistant to ampicillin (100%), tetracycline (53%) followed by kanamycin (41%), erythromycin (30%), and oxacillin (5%) as shown in Table 2. The S. aureus isolates from milk samples (M, A, RM12, 1MB, 2MB and 3MB) had more resistance to the antimicrobial agents as compared to the resistance of sewerage and nasal isolates.

3.2. Antimicrobial analysis of plants extracts

Antimicrobial analysis of plant extracts were checked on multidrug resistant S. aureus. All the multidrug resistant strains and standard ATCC (25923) were tested for MIC against these five potent plant extracts. Maximum inhibition zone was observed in methanolic extract of Psidium guajava (P. guajava), Nigella sativa (N. sativa), Citrus sinensis (C. sinensis), Valeriana jatamansi (V. jatamansi), and Cucurbita pepo (C. pepo). Other extracts such as Acacia nilotica (A. nilotica) and Santalum album (S. album) extracts gave zone of inhibition greater than 10 mm, while all other extracts showed minimum zone of inhibition or no zone as shown in Table 3.

All the multidrug resistant strains and standard ATCC (25923) were tested for MIC against these five potent plant extracts.
extracts: N. sativa (KAL), P. guajava (GUA), C. pepo (CPLM), V. jatamansi (VJAM) and Piper nigrum (PNSM) (P. nigrum) to find out the 50% inhibition of S. aureus. The results of present study showed that the methanolic extract of N. sativa (KAL), resulted in 50% inhibition of ATCC (25923), A, 1MB and 2MB S. aureus strain at a concentration of 0.625 mg/mL, while RM12 and 3MB gave LD₉₀ at a concentration of 2.5 mg/mL and 0.328 mg/mL respectively.

The results of this research represented that the methanolic extract of P. guajava (GUA) gave 50% inhibition of M and 2MB strains of S. aureus at a concentration of 0.625 mg/mL, RM12 and A strains resulted in 50% inhibition at a concentration of 0.325 mg/mL, while 1MB and 3MB S. aureus strains showed LD₉₀ at 1.250 mg/mL.

In this experiment the methanolic extract of C. pepo gave LD₉₀ of M and A stains of S. aureus at the concentration of 1.250 mg/mL and 5.000 mg/mL and RM12 resulted in 50% inhibition at a concentration of 0.625 mg/mL.

Methanolic extract of P. nigrum (PNSM) exhibited LD₉₀ of only ATCC (25923) at a concentration of 0.328 mg/mL, while V. jatamansi (VJAM) extract showed 50% inhibition of RM12, ATCC (25923) and A at a concentration of 5.000 mg/mL, and M gave LD₉₀ at concentration of 0.625 mg/mL as shown in Table 4.

### Table 4

| Sample          | N. sativa | P. guajava | C. pepo | P. nigrum | V. jatamansi |
|-----------------|-----------|------------|---------|-----------|--------------|
| ATCC (25923)    | 0.625     | –          | 0.328   | 5.000     | –            |
| M               | –         | 0.625     | 1.250   | 0.625     | –            |
| RM12            | 2.500     | 0.325     | 0.625   | 5.000     | 5.000        |
| A               | 0.625     | 0.325     | 5.000   | 5.000     | 5.000        |
| 1MB             | 0.625     | 2.500     | –       | –         | –            |
| 2MB             | 0.625     | 0.625     | –       | –         | –            |
| 3MB             | 0.328     | 2.500     | –       | –         | –            |

### 4. Discussion

Staphylococcus is a group of bacteria that can cause a number of diseases as a result of infection of various tissues of the body. Over 30 different types of staphylococci can infect humans, but most infections are caused by S. aureus. In the majority of cases, the bacteria do not cause disease. However, damage to the skin may allow the bacteria to overcome the natural protective mechanisms of the body, leading to infections. S. aureus causes one of the most common types of chronic mastitis. Once established, S. aureus infections do not respond well to antibiotic therapy. Recently published work has shown that 3% of all animals are infected with S. aureus[14]. However, S. aureus represents 10 to 12 percent of all clinical mastitis infections[5].

In the present study, S. aureus strains originated from bovine mastitis were identified phenotypically by some conventional tests. The primary tool for controlling staphylococcal mastitis is antimicrobial therapy. Therefore antimicrobial susceptibility tests were performed according to NCCLS standards to analyze the resistance pattern of S. aureus.

Second part of this study was designed to find out the antibacterial potentials of Pakistani herbal plants against multidrug resistant S. aureus. For this purpose, six multidrug resistant S. aureus strains were isolated from nasal, milk samples. The aim of study was to find out antibacterial agents from indigenous medicinal plants.

As herbal medicines are being used for centuries and botanicals have also become a main point for the identification of new active compounds to treat diseases. Therefore, the focus of this study was to get some knowledge of local plants which are being used for medicinal purposes in Pakistan. Active compounds, derived from plant extracts, are of continuing interest to the pharmaceutical industry.

Anti–bacterial activity of collected herbal extracts was tested against S. aureus. Methanol extracts of 29 different plants were screened for their anti–bacterial activity. Out of them, only five herbal extract (P. guajava, N. sativa, P. nigrum, V. jatamansi and C. pepo) were found to exhibit potential antibacterial activity against S. aureus. Other extracts such as A. nilotica and S. album also gave antibacterial activity against S. aureus to some extent.

Guava leaf tea of P. guajava Linnaeus is commonly used as a medicine against gastroenteritis. Popular Brazilian medicine has long made use of native plants for the treatment of gastrointestinal diseases, as in the case of P. guajava[15]. Chaidate et al. found that P. guajava leaves
extract kills the resistant strains of *S. aureus* using microbroth dilution method and guava leaf extract was able to inhibit all bacterial strains in this study at minimum inhibitory concentration (MIC) between 0.2–51.2 mg/mL [16]. In the present study, the antimicrobial activity of *P. guajava* showed the inhibitory effect at a concentration of 40 mg/mL on all six multidrug resistant strains of *S. aureus* isolated from milk *i.e.* M, A, RM12, 1MB, 2MB and 3MB. While methanolic extract of *P. guajava* did not show inhibitory effect on standard stain of *S. aureus* ATCC (25923). The MIC value of methanolic extract of *P. guajava* was in between 0.3–2.5 mg/mL.

*N. sativa* is a herbaceous plant found in the Middle East, Europe and Western and Middle Asia. Its seeds have a great medicinal potential and have been reported to exhibit many pharmacological effects that include anti-parasitic, antibacterial, antifungal, antiviral, antioxidant and anti-inflammatory activities [17]. Hannan et al. found that strains of methicillin-resistant *S. aureus* were sensitive to *N. sativa* extract at a concentration of 4 mg/disc while the extract had an MIC range of 0.2-0.5 mg/mL. Our study was therefore designed to evaluate this aspect of *N. sativa*. The results of disc diffusion assay demonstrated that strains of multi-drug resistant *S. aureus* were completely inhibited at 40 µg/mL on 6 mm disc and gave MIC value between 0.3–2.5 mg/mL.

*C. pepo* (pumpkin) belongs to the family Cucurbitaceae which includes cucumbers, melons, squash and gourds. Pumpkin is used as an emollient to soften the dryness of the skin and pimples spots. It is used in treating bladder disorders, stomach upsets, intestinal worms. Chonoko and Rufai 2011 described that antibacterial activity of *C. pepo* showed that its ethanol extract was active against *S. aureus* and *Salmonella typhi* (7mm) [19]. The methanol extract was active against *S. aureus* but inactive against *Salmonella typhi*. This study sought to screen *C. pepo* to determine the antibacterial activity of the plant extracts against *S. aureus*. The results of antibacterial activity showed that methanolic extract of *C. pepo* was active against three mastitis strains of *S. aureus* (M and RM12 and A) but has not shown any zone of inhibition against ATCC (25923) strain which is of clinical origin. The zone of inhibition for M and RM12 and A were (14.50±0.70) mm, (11.00±1.41) mm and (13.50±0.70) mm respectively and exhibited MIC value at a concentration between 0.625–5.000 mg/mL. Saponins were present in the extract which has been found to be used as anti-inflammatory and anti-oxidant agents.

*V. jatamansi* is also known as *Valeriana wallichii* is an Indian plant which belongs to the family Valerianaceae. This plant is never used as antimicrobial against *S. aureus*. Here we used methanolic extract of axial part of this plant against multidrug resistant *S. aureus* and it gave a very good zone of inhibition. The zone of inhibition for mastitis *S. aureus* isolates *i.e.*, A, M, RM12, 1MB, 2MB, 3MB and clinical isolated *S. aureus* ATCC (25923) was (10.00±0.70) mm, (15.50±0.70) mm, (11.50±2.12) mm, (10.00±0.00) mm, (10.00±2.80) mm and (14.50±0.70) mm respectively and showed MIC values in between 0.625–5.000 mg/mL.

*P. nigrum* is used to treat asthma, chronic indigestion, colon toxins, obesity, sinus, congestion, fever. It has been shown to have antimicrobial activity. Chaudhry, Tariq et al. investigated the effect of *P. nigrum* on clinically isolated strains of *S. aureus* and found that both aqueous and methanolic extract of *P. nigrum* gave 23 mm zone of inhibition at the concentration of 10 µL/disc [18]. And their results are completely overlapping the results of this research. In this study only ATCC (25923) stain gave zone of inhibition (21.00±2.00) mm, and a very little zone was appeared in M bacteria *i.e.* (9.00±1.41) mm. *P. nigrum* extract resulted in 50% inhibition at a concentration of 0.328 mg/mL.

On the basis of results presented herein, it is theoretically possible that extracts of *P. guajava*, *P. nigrum* seed, *V. jatamansi*, *C. pepo* and *N. sativa* may play a role in the treatment *S. aureus* infection. This data also suggest that therapeutic induction of extracts might represent an alternative approach for the treatment of mastitis and nosocomial infections caused by *S. aureus* or this study may leads to the development of more potent drugs for *S. aureus* infections.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

**Background**

*S. aureus* multidrug resistant is a serious problem in the concern of mastitis treatment. Several medicinal plants are capable of inhibit microbial growth and are frequently used by popular knowledge to treat infections.
Research frontiers

The present work highlights the emergence of multi-resistant S. aureus in mastitis cases and demonstrates the efficacy of plant extracts against these isolates.

Related reports

A number of medicinal plants are usually tested for their antimicrobial potential against mastitis pathogens, specially S. aureus. An example is the work of Kalayou et al. (2012) doi: 10.1016/S2221-1691(12)60088-4. However, the emerging of multi-resistant S. aureus increases the need for new effective drugs against these agents.

Innovations and breakthroughs

In the present study, authors have demonstrated the antimicrobial activity of some plant extracts selected due to the popular knowledge about their medicinal properties against multi-resistant S. aureus.

Applications

This scientific study suggest the use of the plants P. guajava, N. sativa, P. nigrum, V. jatamansi and C. pepo as sources of antimicrobial agents against some strains of multiresistant S. aureus.

Peer review

The article content is valid for publication because it addresses the importance of alternatives to control multiresistant S. aureus. This theme has been recurring in the literature because it is a current problem in both the field of animal and human health. So, it can be considered for publication due to its theoretical value.

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