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PHYTOCHEMICAL SCREENING AND ANTIMICROBIAL ACTIVITIES OF EVER GREEN MEDICINAL PLANT *ANNONA MURICATA* - "AN EDIBLE VACCINE"

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ABSTRACT

Medicinal plants are now getting more attention because they have potential of myriad benefits to the society. These plants may promote host resistance against infection by re-stabilizing body equilibrium and conditioning the body tissues. Chemically, medicinal plants may have secondary metabolites like alkaloids, glycosides, steroids or other groups of compounds which have marked pharmaceutical action as antimicrobial, antioxidant, anticancer, antimalarial, antidiabetic, *etc*. These metabolites are required after because they are known to exhibit number of biological activities that promotes health effects. The screening of plant extracts and their products for antimicrobial activity has shown that higher plants represent a potential source of novel antibiotic prototypes. The selection of crude plant extracts for screening programs is potentially more successful in initial steps than the pure compounds. Once such a plant is Graviola*(Annona muricata*) which belongs to the family of Annonaceae is an evergreen tree species used as traditional medicines known to possess medicinal uses, but the biological and pharmacological properties are unexplored. There is no systematic work that has been undertaken fruit on this plant and this is the first report of antimicrobial activities ethanolic extract of fruit of *Annona muricata*. The present study has been formulated to understand the *in vitro* phytochemical and antimicrobial properties elicited by *Annona muricata*. The analysis revealed that *Annona muricata* needs further research on toxicological aspects to develop safe drug.

KEYWORDS: Secondary metabolites, *Annona muricata*, antimicrobial activity, ethanolic extract, Phytochemical analysis.

INTRODUCTION

Traditional systems of medicine are popular in developing countries and up to 80% of the population relies on traditional medicines or folk remedies for their primary health care need. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects. The unique and complex structures of natural products cannot be obtained easily by chemical synthesis. A number of plants in the world have been used in traditional medicine remedies.[1] Nearly, all cultures and civilizations from ancient times to the present day have depended fully or partially on herbal medicine because of their effectiveness, affordability, availability, low toxicity and acceptability.[2]

Phytochemicals play a vital role against number of diseases such as asthma, arthritis, cancer etc. unlike pharmaceutical chemicals these phytochemicals do not have any side effects. Since the phytochemicals cure diseases without causing any harm to human beings these can also be considered as "man- friendly medicines". Plants have played a remarkable role in healthcare since the ancient times. Traditional plant based medicines still exert a great deal of importance to people living in developing countries and also leads to the discovery of new drug candidates.[3]

Infectious diseases are the major cause of morbidity and mortality worldwide. The number of multidrug resistant microbial strains and the appearance of strains which reduced susceptibility to antibiotics are continuously increasing. Such increase has been attributed to indiscriminate use of broad spectrum antibiotics, immunosuppressive agents, intravenous catheters organ transplantation and ongoing epidermis of human immunodeficiency virus (HIV) infections. This situation provided the impetus to the search for new antimicrobial substances from various source like medicinal plants. Plant based natural constituents can be derived from any part of the plants like bark, leaves, fruits, seeds etc.[4] i.e. any part of the plant may contain active compound. In light of the recent emergence of bacteria which are resistances to multiple antimicrobial drugs posing a challenge for the treatment of infections.[5] The need to

discover new antimicrobial substance for use in combating such microorganisms becomes pertinent.

Annona muricata (common Spanish name: guanábanaa) is a species of the genus *Annona* of the custard apple tree family, Annonaceae, which has edible fruit. The fruit is usually called soursop due to its slightly acidic taste when ripe. *A. muricata* is native to the Caribbean and Central America but is now widely cultivated and in some areas, becoming invasive in tropical climates throughout the world.

Related species include cherimoya (*A. cherimola*) and sugar-apple (*A. squamosa*); paw paw (*Asimina triloba*) is also in the family. Other common names include graviola and guanábana (sometimes shortened to guanába). Due to uniqueness of curing different ailments, the whole fruits of a *Annona muricata* was selected for the study. Hence the present investigation was carried out to determine the possible phytochemical components from *Annona muricata* and to analyze the antimicrobial activity against selected microorganisms.

MATERIALS AND METHODS

Plant Collection and Identification

Annona Muricata used in the study was identified and the reference material has been kept under reference ACAS/SC/05/16-17. Fresh whole fruit was collected randomly from the region of in around Kolli Hills, Tamilnadu. Fresh fruit was air dried and then homogenized to fine powder and stored in air tight bottle. The extracts were then, dried in vacuum and stored in a refrigerator. Yield of extract 50grams of whole fruit powder yielded 6.8g.

Phytochemical Screening Analysis of *Annona Muricata*

Qualitative and Quantitative Phytochemical Analysis

Annona muricata extracts obtained by the above method was subjected to qualitative analysis for the presence of Phenolic groups, Glycosides, Alkaloids, Flavonoids, Tannins, Terpenoids, Saponins, Oils and gums as described by the method of Trease and Evans (2002), Sofowara, (2008) and also as specified in the book of Practical Pharmacognosy.[7]

❖**Detection of Alkaloids:** Extracts were dissolved individually in dilute hydrochloric acid and filtered. The filtrates were used to test the presence of alkaloids.

- **Mayer's test**: Filtrates were treated with Mayer's reagent. Formation of a yellow cream precipitate indicates the presence of alkaloids.
- **Wagner's test**: Filtrates were treated with wagner's reagent. Formation of brown/ reddish brown precipitate indicates the presence of alkaloids.

❖**Detection of Flavonoids**

- **Lead acetate test**: Extracts were treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of flavonoids.
- **H_2SO_4 test**: Extracts were treated with few drops of H_2SO_4. Formation of orange colour indicates the presence of flavonoids.

❖**Detection of Steroids:** 2ml of acetic anhydride was added to 0.5g of the extracts, each with 2ml of H_2 SO_4. The color changed from violet to blue or green in some samples indicate the presence of steroids.

❖**Detection of Terpenoids**

- **Salkowski's test:** 0.2g of the extract of the whole plant sample was mixed with 2ml of chloroform and concentrated H_2SO_4 (3ml) was carefully added to form a layer. A reddish brown coloration of the inner face was indicates the presence of terpenoids.

❖**Detection of Anthraquinones**

- **Borntrager's test:** About 0.2g of the extract was boiled with 10% HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10% NH_3 were added to the mixture and heated. Formation of pink color indicates the presence anthraquinones.

❖**Detection of Phenols**

- **Ferric chloride test**: Extracts were treated with few drops of ferric chloride solution. Formation of bluish black colour indicates the presence of phenol.

- **Lead acetate test**: Extract was treated with few drops of lead acetate solution. Formation of yellow colour precipitate indicates the presence of phenol.

❖**Detection of Saponins:** About 0.2g of the extract was shaken with 5ml of distilled water. Formation of frothing

(appearance of creamy miss of small bubbles) shows the presence of saponins.

❖**Detection of Tannins:** A small quantity of extract was mixed with water and heated on water bath. The mixture was filtered and ferric chloride was added to the filtrate. A dark green color formation indicates the presence of tannins.

❖**Detection of Carbohydrates:** Extracts were dissolved individually in 5ml distilled water and filtered. The filtrate was used to test the presence of carbohydrates.

❖**Detection of Oils and Resins:** Test solution was applied on filter paper. It develops a transparent appearance on the filter paper. It indicates the presence of oils and resins.

Antimicrobial Activity

Microbial Strains Used For Assay: Totally Eight bacterial strains and two fungal strains were used throughout investigation. All the bacterial and fungal cultures were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The bacteria used were Bacillus subtillis, Staphylococcus aureus, Enterococccus faecalis, Klebsiella pneumoniae, Micrococcus luteus, Shigella flexneri, Salmonella typhi and Proteus vulgaris. The fungal strains used were Aspergillus oryzae and Candida albicans. The young bacterial broth cultures were prepared before the screening procedure.

Antibacterial Assay

Preparation of Inoculums: Stock cultures were maintained at 4^{o}C on slopes of nutrient agar. Active cultures of experiment were prepared by transferring a loopful of cells from the stock cultures to test tube of Muller-Hinton broth (MHB) for bacteria that were incubated without agitation for 24 hrs at 37^{o}C. The cultures were diluted with fresh Muller-Hinton broth to achieve optical densities corresponding to 2.0 X 10^{6} colony forming units (CFU/ml) for bacteria.

Antibacterial Assay: The well diffusion method was used to screen the antimicrobial activity. *In vitro* antimicrobial activity was screened by using Muller Hinton Agar (MHA) obtained from Himedia (Mumbai). The MHA plates were prepared by pouring 15 ml of molten media into sterile petriplates. The plates were allowed to solidify for 5 minutes and 0.1% inoculums suspension was swabbed uniformly and the inoculums were allowed to dry for 5 minutes. Wells were cut and 20 µl of the different concentration of ethanolic extract of *Annona muricata* were added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well (NCCLS, 1993). Chloramphenicol disc was used as a positive control.

Antifungal Assay: Totally two fungal strains were used throughout investigation. All the fungal cultures were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The young fungal broth cultures were prepared before the screening procedure.

Antifungal activity: Antifungal activity was measured using methods of well diffusion plates on agar. In order to test the antifungal activity, the fractions of different concentration of plant extract were dissolved in ethanol. 20 mL of Sabouraud Dextrose Agar was poured into each 15 cm Petri dish. C. albicans and A. oryzae were grown in Sabouraud Dextrose Broth at 27° C for 48 h. Growth was adjusted to OD (600 nm) of 0.1 by dilution with Sabouraud Dextrose Broth . Then, Wells were cut and 20 µl of the different concentration of ethanolic extract of *Annona muricata* were placed on agar to sample (1 mg/mL). 100 units of Fluconazole, obtained from a local pharmacy, were used as a positive control. Inhibition zones were determined after incubation at 27° C for 48 hrs.

RESULTS AND DISCUSSION

Table. 1. Qualitative Analysis of the Phytochemicals in the *Annona Muricata* with Various Solvent Extracts.

S. No	Bioactive compounds	Aqueous	Methanol	Ethanol	Petroleum ether	Chloroform
1	Alkaloid	+	+	++	ND	ND
2	Flavonoid	++	++	+++	+	+
3	Triterpenoid	ND	++	+	ND	+
4	Phenolic compound	+	+	+++	+	ND
5	Protein	ND	+	+	+	ND
6	Carbohydrates	+	+	++	ND	ND
7	Saponin	+	++	+++	-	++
8	Steroids	+	+	+	+	ND
9	Glycosides	+	+	+	ND	+
10	Amino acid	ND	+	+	ND	ND
11	Tannin	+	++	++	+	+
12	Oil	ND	ND	ND	ND	ND
13	Gums & Musilage	ND	ND	ND	ND	ND
14	Chlorogenic compound	ND	ND	ND	ND	ND

+++ = high; ++ = moderate; + = low; ND = not detectable

The phytochemcial characters of *Annona muricata* investigated are presented in Tables 1. In the present investigation, preliminary phytochemical screening done in the various extracts aqueous, ethanol, methanol, chloroform and petroleum ether of *Annona muricata* showed the presence of phytochemical constituent's namely alkaloids, flavonoids, glycosides, saponins, steroids, tannins, phenols, triterpenoid, and the absence of fixed oils and fats, gums and chlorogenic compounds. Phytochemical test showed that substance contained soursop showed higher phytoconstituents like flavonoid, saponin, alkaloid, tannin and steroid content. From the above Table 1 results it was inferred that broad range of secondary metabolites were present in the ethanolic extract of *Annona muricata*. Hence, it is utilized for further experimental analysis.

This result relevant with study by Vimala et al which said that ethanolic extract of soursop leaves has secondary metabolite such as flavonoid, tannin, alkaloid, saponin and steroid.[8]

From the above investigation comprehensively validate the presence of therapeutically important and valuable secondary metabolites like alkaloids, flavonoids, phenols, tannins and steroids in fruits of *Annona muricata*

Table. 2. Quantitative Estimation of Bioactive Constituents in Ethonolic Extract of *Annona Muricata.*

Name of the Phytoconsituent	%
Flavonoids	8.72 ± 0.60
Ascorbic acid	43.67 ± 1.81
Alkaloids	1.42 ± 0.07
Tannins	0.13 ± 0.01

Phytochemical screening of the plants showed the presence of alkaloids, flavonoids, glycosides, phenolic compounds, phytosterols, quinones, saponins, steroids and terpenoids. The ethanolic extract shows high amount of Ascorbic acid 43.67 ± 1.81 %, Flavonoids 8.72 ± 0.60%, Alkaloids 1.42 ± 0.07 % and Tannins 0.13 ± 0.01% respectively.

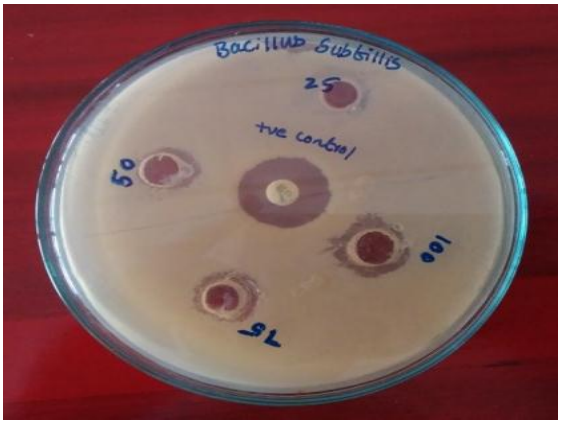
Table. 2. Antimicrobial Activity of Ethanolic Extract of Fruits of *Annona Muricata.*

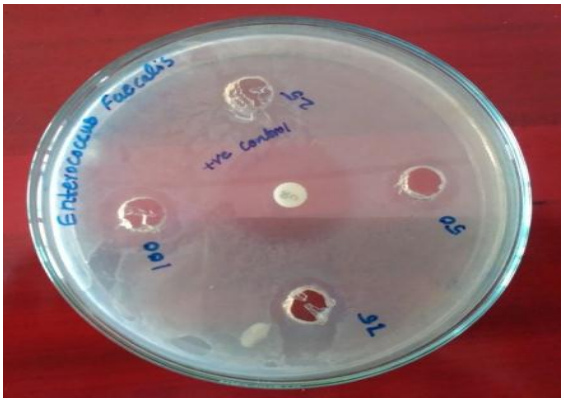
S. No	Microorganisms	C	100 µl	75 µl	50 µl	25 µl
Gram Positive Bacteria						
1	*Enterococccus faecalis*	27	17	15	11`	10
2	*Bacillus subtillis*	21	18	14	12	09
3	*Micrococcus luteus*	20	14	11	10	07
4	*Staphylococcus aureus*	26	17	12	11	08
	Gram Negative Bacteria					
1	*Shigella flexnari*	18	15	13	12	10
2	*Klebsiella pneumonia*	20	16	14	10	08
3	*Salmonella typhi*	19	14	12	11	07
4	*Proteus vulgaris*	18	13	11	10	08
Fungal						
1	*Candida albicans*	25	11	10	08	05
2	*Aspergillus oryzae*	23	10	08	06	04

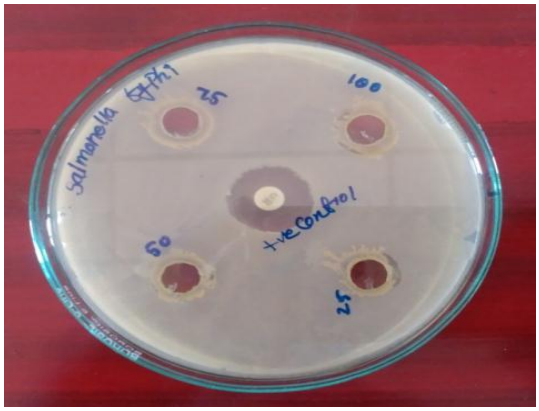
Antibacterial effects of the ethanolic extract of *A.muricata* (Table -2 and Figure-1) more potential against *Bacillus subtillis, Enterococccus faecalis, Klebsiella pneumonia, Shigella flexnari* and moderate activities against *Salmonella typhi, Micrococcus luteus, Proteus vulgaris*. Minimum activity against fungal like *Candida albicans, Aspergillus oryzae* suggest that they may possess remarkable therapeutic action in the treatment of infectious diseases. Among the 5 Gram-positive bacteria assayed, *Bacillus subtillis, Enterococccus faecalis* showed maximum zone of inhibition with 17 mm, whereas Gram-negative bacteria such as *Klebsiella pneumonia* showed 16 mm the same value of inhibition at the concentration of 10 mg /ml. Antifungal assay showed that the *Candida albicans* 11 mm was more susceptible to ethanol extract of *A.muricata*.

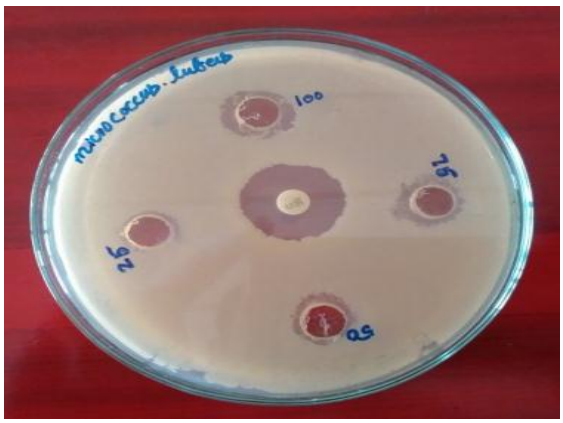
Increasing, evidence indicates that, *Annona muricata* and Annona based food products contain significant leaves of natural antimicrobial and may provide health benefits to consumers in addition to general nutrients and energy.[9]

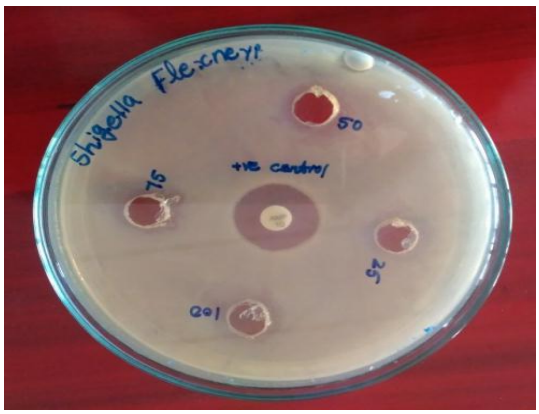
GRAM Positive

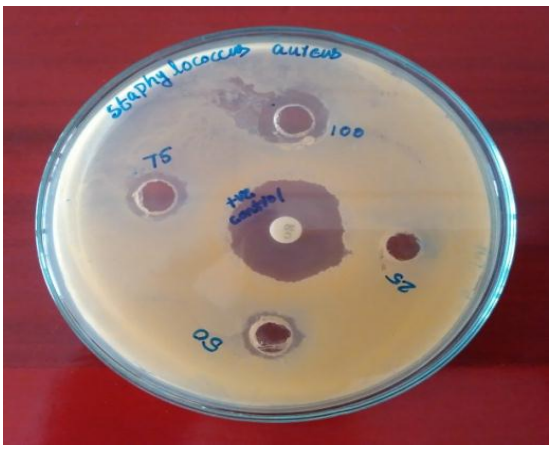


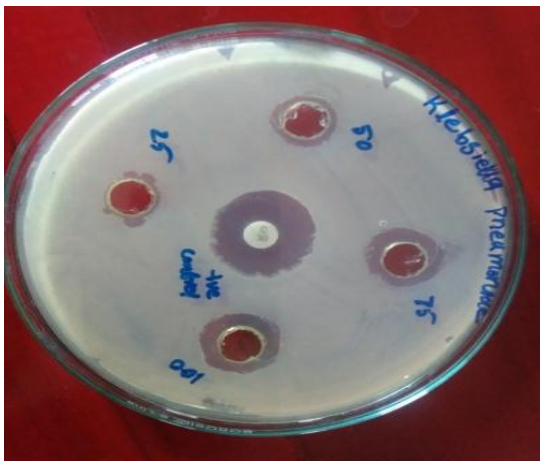




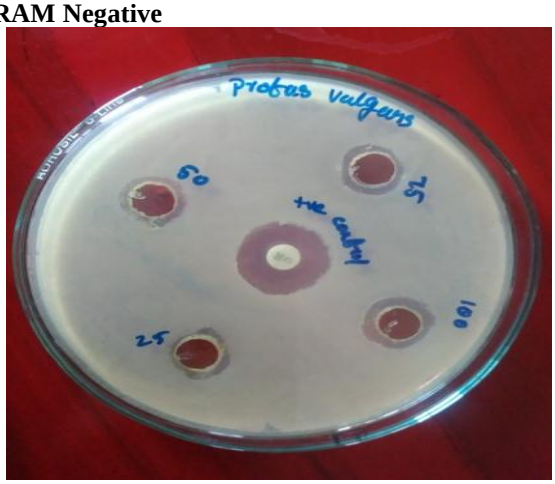




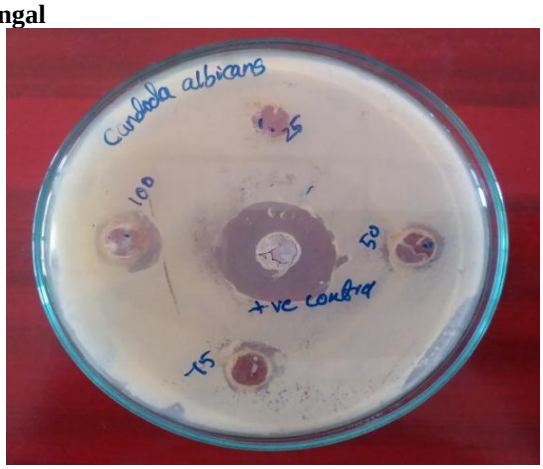




GRAM Negative



Fungal



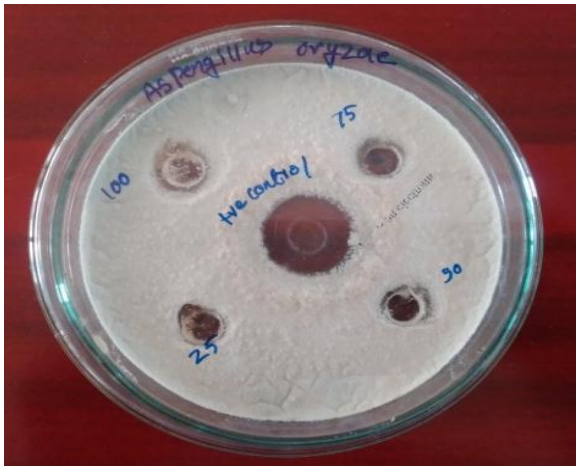


Figure. 1. Photographic Representation of Antimicrobial Properties of *Annona Muricata.*

Many commonly used natural products like neem stick, pome-granate and tulsi have been tested for their antibacterial pro-perties. Most of these plants used for traditional medicines are grown locally and have been used for centuries as medicine in those regional areas. Also, resistance of pathogenic bacteria to currently used antibiotics and chemotherapeutics has increased the global requirement for alternative safe, efficacious and cost-effective treatment options for infections, particularly in developing countries.[10]

In this antimicrobial assay, the zone of inhibition was found to dose-dependent. The compounds responsible for antimicrobial activity have to be isolated which may reduce the doses of extract used as antimicrobial agent. The use of Soursop extract on microorganisms has a strong traditional foundation; many countries in the world use this extract for treatment of various diseases. In countries like Peru, Brazil and Togo the extracts have been used for various treatments such as liver disorders, diarrhoea, dysentery, fevers, hypertension, sores, internal ulcers and diabetes.[11]

CONCLUSION

A. muricata is a popular tropical tree, and a wealth of phytochemical investigations has been conducted for this fruit. In addition to being a key source for the food industry and an indigenous medicinal plant, *A. muricata* is confirmed to possess a wide spectrum of biological activities. Among all former studies on this plant, the most promising activities are found to be its anticancer, antiparasitic and insecticidal activity. Because the majority of the previous studies were focused on the biological activities of the plant extract, further investigations on the biochemical and physiological functions of active compounds and the detailed mechanisms underlying these activities are completely essential for the development of pharmaceutical and agricultural products. These promissory extract open the possibility of finding novel clinically effective antibacterial compounds.

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