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Cytotoxicity by Brine Shrimp Lethality Test and Potential *Rhinachantus nasutus* (L.) Kurz Stem Bark Ethanol Extract as Anti-Inflammatory and Antimicrobial

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Abstract

The ethanol extract of Rhinachantus nasutus (L.) Kurz stem bark (RNSB) was prepared using ultrasonication. This extract has a high total phenolic content, strong antioxidant activity, and actively inhibits alpha-glucosidase activity. On the other hand, the possible cytotoxic, anti-inflammatory, and antibacterial effects of the ultrasonic extract have not been reported. The purpose of this study was to investigate the cytotoxicity of an ethanol extract of RNSB using the Brine Shrimp Lethality Test (BSLT) method with Artemia salina larvae as the test organism. In addition, anti-inflammatory activity was evaluated using the protein denaturation inhibition method, and antibacterial activity was evaluated using the paper disc method against Escherichia coli and Bacillus subtilis. This study showed that RNSB ethanolic extract had a strong cytotoxic effect with an LC50 of 10.93 ppm. The anti-inflammatory effect of this RNSB extract is also very strong, with an IC50 value of 3.00 ± 0.009 mg/L. In contrast, the RNSB extract was not effective against E. coli and B. subtilis bacteria. These findings suggest that R. nasutus stem bark may have anti-inflammatory and anti-cancer properties.

Keywords: antibacterial, anti-inflammatory, cytotoxicity, RNSB

1. Introduction

Rhinachantus nasutus (L.) Kurz is a species of shrub that may frequently be found growing in the shadow along the sides of roadways. This kind of plant can be found growing wild across a significant portion of Southeast Asia, India, and China [1-2]. The RNSB ethanolic extract has been shown in previous research to have high total phenolic content (approximately 677 mgGAE/g sample, strong antioxidant activity (IC50 value of 18.43 mg/L), and to be active as an alpha-glucosidase inhibitor (IC50 value of 10.95 mg/L) [3]. Antioxidants derived from plants are crucial in the battle against degenerative disorders caused by oxidative stress [4-5]. Cancer and inflammatory illnesses are examples [6-7].

The biological process known as inflammation is generated when vascular tissue is subjected to harmful stimuli such as viruses, damaged body cells, or irritants. Inflammation is a complex response that the body goes through in response to these noxious stimuli [8]. Inflammation is a hallmark of the state that is connected with chronic illness, which is marked by discomfort, dysfunction in organs and tissues, edema, and redness [9-10]. There is a wide variety of treatments available to ease the discomfort caused by inflammation; however, conventional medicine has not been shown to be either effective or safe enough to be regarded a viable choice. As a consequence of this, there is an urgent want for a strong and effective anti-inflammatory medicine, in particular for the management of chronic conditions [11]

Unresolved, uncontrolled, or chronic inflammation can be a contributing factor in the development of a variety of diseases, including cancer [12]. Cancer risk is increased and tumor growth is stimulated throughout the process by inflammation. An inflammatory tumor microenvironment is the result of coordinated interactions between cancer cells and the stromal and inflammatory cells that surround them [13]. Cancer is one of the main causes of death around the globe and is a significant contributor to mortality rates in developed nations. Cytotoxicity test was conducted using the procedure for the Brine Shrimp Lethality Test (BSLT), with Artemia salina serving as the test organism. It is normal practice to conduct preliminary tests on leach larvae in order to

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determine the anti-tumor and anti-cancer effectiveness of a substance [14]. Numerous studies have found evidence supporting the hypothesis that there is a link between certain types of bacteria and cancer [15].

Antibiotics are the drugs of choice for treating bacterial infections. Antibiotic resistance arises for several reasons, including the overuse of antibiotics, inadequate dosing, and therapy discontinuation prior to bacterial eradication [16]. As a result, there is an urgent requirement for the development of new medicinal compounds with qualities that allow them to be effective against inflammation and cancer, in addition to combating the problem of bacterial resistance.

This study examined the cytotoxic capability of an ethanolic RNSB extract using BSLT. An ethanolic extract was also tested for anti-inflammatory and antibacterial properties. This study used a 70% ethanol extract of RNSB, which has been produced by ultrasonic extraction and has been reported in previous studies [3].

2. Experimental

2.1. Preparation and extraction of simplicia

As was done in earlier research, the samples of RNSB (specimen number 1079/IPH.1.01/IF. 07/XI/2020) used in this investigation were prepared by ultrasonic extraction using 70% ethanol as the solvent, which has been reported in our previous publication [3].

2.2. Cytotoxicity test

Test for cytotoxicity carried out with the Brine Shrimp Lethality Test (BSLT) procedure with *Artemia salina* serving as the test organism [12]. In a sealed container, 30 mg of brine shrimp eggs from *A. salina* are added to the saltwater. Putting an air hose in the bottom of the container helps the eggs hatch. After 24 hours, the eggs of *A. salina* will hatch and turn into larvae. Then, we took 10 larvae from each species and put them into containers with sample solutions of 0, 10, 100, and 250 mg/L. The *A. salina* larvae in both the samples and the controls had obviously died after 24 hours. Larvae of *A. salina* can be considered dead if they haven't moved in more than a few seconds. The data was linearly regressed after the proportion of *A. salina* larvae that perished was calculated, and the probit value was located via a search of the probit table.

2.3. Anti-inflammatory test

Bovine serum albumin (BSA) was employed in a protein denaturation inhibition assay to determine whether or not it possessed anti-inflammatory capabilities [17]. Three separate samples totaling 80 µL, 120 µL, and 160 µL were taken from a 1000 mg/L extract solution and subsequently transferred to a 5 mL volumetric flask. Then, a 0.2% BSA solution in tris buffered saline (TBS) was used to dissolve it. The solution was first incubated at room temperature for 30 minutes before being heated at 72 °C in a water bath for 5 minutes. The solution was left out at room temperature for 25 minutes. In order to measure absorption, a visible spectrophotometer at 660 nm was used. The experiment was repeated five times with a blank control and also three times with a positive control of diclofenac sodium at concentrations of 0.5, 0.75, and 1.0 mg/L.

2.4. Antibacterial test

Paper discs of approximately 6 mm in diameter were used in the disc diffusion method to assess the antibacterial activity of *Escherichia coli* and *Bacillus subtilis*. A total of two rounds of testing were performed on the antibacterial agents. When the paper discs were finished, they were placed on a culture medium (Mueller Hinton Agar) that had been injected with a pathogen solution and then submerged in samples with a concentration of 100 mg/L. During the incubation procedure, two cycles of 24 hours were carried out at 37 degrees Celsius. The size of the inhibitory zone was plotted out and measured on the paper disc [18, 19].

3. Results and discussion

3.1. Cytotoxicity activity

The BSLT technique was applied in order to evaluate the cytotoxicity of the ethanol extract of *R. nasutus* stem bark at the following concentrations: 0; 10; 100; 250; 500 and 1000 mg/L. In Table 1, we present the number of *A. salina* larvae, as well as the percentage of those larvae that died as a result of the various test concentrations. Using this information, a statistical probit analysis is performed, yielding a plot showing how the probit value relates to the logarithm of the concentration (as shown in Figure 1).

Table 1. Results of cytotoxicity tests on ethanolic extract of RNSB

Concentration (mg/L)	Log Concentration	Total Larva Test	Mortality	% Mortality	Probit Value
0	0	10	0	0	0
10	1	10	1	10	3.72
100	2	10	2	20	4.16
250	2.398	10	5	50	5.00
500	2.699	10	7	70	5.52
1000	3	10	9	90	6.28

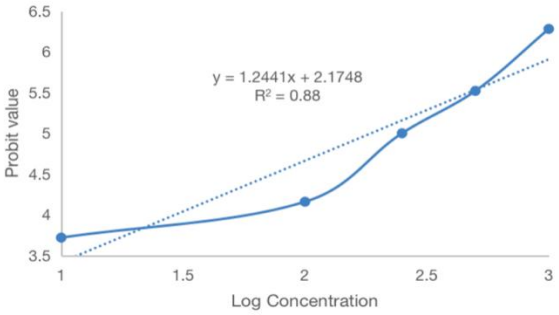

Fig. 1. Chart of profitability of ethanol extract of RNSB

The LC_{50} value of the ethanol extract of RNSB was determined to be 186.59 mg/L, and the line equation y = 1.2441x + 2.1748 was obtained from the values determined. This suggests that the extract is toxic, as it was responsible for the deaths of 50% of the test animals at quantities of less than 1000 ppm [20]. It has been discovered that there is a correlation between the antiproliferative activity of the extract against cancer cells and the cytotoxicity of the extract against cancer cells. The capacity of a drug to cause death in cancer cells is known as its cytotoxicity. Power to inhibit the proliferation of cancer cells is referred to as antiproliferative power. According to the results of the BSLT test, the ethanol extract that was taken from the stem bark of the *R. nasutus* plant, which is known to be toxic, has the ability to inhibit the proliferation of cancer cells [21, 22].

Based on this information, further in vitro and in vivo tests can be carried out on the RNSB to find out more about its potential as an anticancer drug. Previous studies have shown that the ethanolic extract of RNSB contains a sizeable amount of total phenol [3]. The class of compounds known as polyphenols is the one that can be discovered in the greatest number of different plant species. Based on the structure of the aglycoside, polyphenolic compounds can be further split into categories consisting of phenolic acids, flavonoids, polyphenol amides, and other polyphenols. These different types of polyphenols each have their own set of distinguishing characteristics [23]. Studies have been conducted on phenolic compounds in order to examine the antioxidant, anti-carcinogenic, alpha-glucosidase activity inhibitory, anti-inflammatory, and free radical scavenging properties that these compounds are thought to possess [24,25, 26].

3.2. Anti-inflammatory activity

For the purpose of this study, we used the anti-inflammatory test of protein denaturation inhibition. As seen in Williams' research, blocking BSA protein denaturation can serve as a preliminary screening for anti-inflammatory action before anti-inflammatory tests are conducted on experimental animals [27]. Heating BSA causes a change in its secondary and tertiary structures, which results in the denatured state. This shows that albumin is damaged by heat, which triggers a negative immune response. Inflammatory responses become difficult for the body to regulate as a result [28, 29]. The body's proteins are vulnerable to denaturation due to the production of free radicals, which results in the release of inflammatory mediators and subsequently causes inflammation [30].

Table 2. Results from studies involving anti-inflammatory medications

Sample	Concentration (mg/L)	% Inhibition	IC_{50} (mg/L)
Sodium Diclofenac	0.50	30.61 ± 1.4	0.88 ± 0.02
	0.75	40.82 ± 1.8	
	1.00	57.14 ± 1.3	
Ethanolic Extract	1.00	16.48 ± 0.4	3.00 ± 0.009
	2.00	32.97 ± 0.8	
	4.00	67.03 ± 0.8	

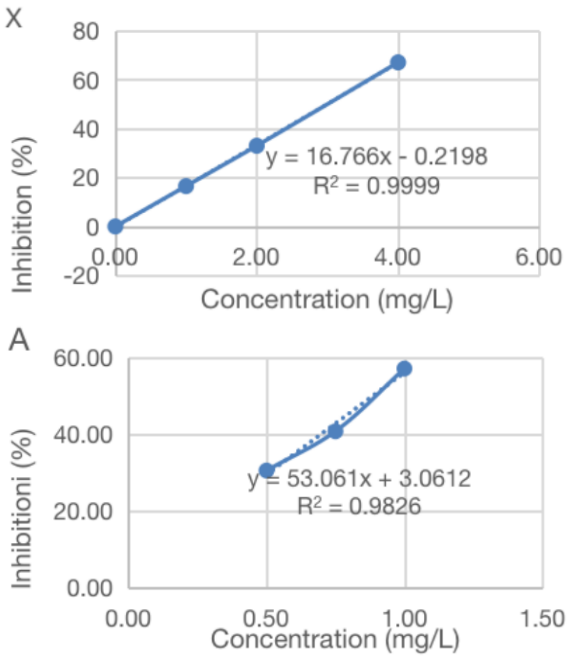

Fig. 2. A graph depicting the link between the percent of inhibition achieved and the concentration used in the calculation of the IC_{50} value for sodium diclofenac (A) and ethanolic RNSB extract (X)

When 1, 2, and 4 mg/L of an ethanol extract of RNSB were used, the percentage of inhibition was 16.48 ± 0.4, 32.97 ± 0.8, and 67.03 ± 0.8, respectively. Diclofenac sodium solution at a concentration of 0.5, 0.75, and 1.0 mg/L mg/L showed a percentage inhibition of 30.61 ± 1.4, 40.82 ± 1.8, and 57.14 ± 1.3, respectively (as shown in Table 2). Sodium diclofenac or stem bark extract concentration is revealed to have a linear relationship with inhibition percentage in Figure 2 (y = 53.061x + 3.0612 and y = 16.766x - 0.2198, respectively). Calculations showed that while

sodium diclofenac had an IC_{50} of 0.88 ± 0.02 mg/L, the RNSB extract had an IC_{50} of 3.00 ± 0.009 mg/L.

The significant anti-inflammatory impact of nasutus stem bark extract is consistent with its high total phenolic content and its high antioxidant effect [3]. Phenolic compounds are found naturally in products with antioxidant effects. In inflammatory autoimmune diseases like glomerulonephritis, denatured albumin serves as an antigen. Other examples of such reactions include type III hypersensitivity and serum sickness. Thus, an agent that prevents albumin degradation or stabilizes it by more than 20% might be assumed to have anti-inflammatory effects and subjected to further anti-inflammatory testing [27, 31, 32]. Based on this information, further in vitro and in vivo tests can be carried out on the RNSB to find out more about its potential as an anti-inflammatory drug.

3.3. Antibacterial activity

Table 3 presents the findings of an analysis of the RNSB that was subjected to an examination of its ability to inhibit the growth of bacteria. In this particular experiment, the strains of *E. coli* and *B. subtilis* bacteria were utilized. Antibacterial activity can be evaluated by measuring the size of the zone of inhibition that surrounds the paper disc. Through the use of the agar diffusion method, the inhibitory zone could be viewed. The extract of the test material was evaluated for its antibacterial activity against *E. coli* and *B. subtilis* at a concentration of 0.11 mg/L; the results are depicted in Figure 3. The resulting inhibition zones were 0.60 and 1.00 mm in diameter, respectively, while the amoxicillin used as a standard was 1.55 and 3.55 mm in diameter.

Table 3. The results of an antibacterial test performed on an standard drug amoxicillin and ethanolic extract of RNSB against *E. coli* and *B. subtilis* were promising

Sample	Bacteria	Average (mm)	The activity of the antimicrobial inhibition zone
Amoxicillin	*E. coli*	1.55	
	B. subtilis	3.35	
Ethanolic extract	*E. coli*	0.60	Weak
	B. subtilis	1.10	Weak

The results of the tests designed to determine whether or not an antibacterial agent is effective against *E. coli* and *B. subtilis* germs show that the activity of the agent is weak. How far apart these bacteria's inhibition zones are depends on how much their environment affects them [33]. The antibacterial effect that *B. subtilis* possesses is significantly more potent than that of *E. coli*. The reason for this is that each of its essential components has a slightly unique cell pattern. The surface morphology of gram-positive bacteria, such as *B. subtilis*, is completely different from that of gram-negative bacteria, like *E. coli*. The outermost layer of gram-positive bacteria is composed of negatively charged teichoic and teicuronic acids, while the cell wall is composed primarily of peptidoglycan (90%). It has been found that the outermost layer of the cell wall in gram-negative bacteria contains between 5 and 20% peptidoglycan as a component of its make-up. This was an interesting discovery. This layer can also be referred to as the lipopolysaccharide layer, and it is located in the core of the structure. It is the second lipid layer, and another name for it is the lipopolysaccharide layer. The three components that make up this layer are phospholipids, polysaccharides, and proteins. This layer is made up of these three components [34].

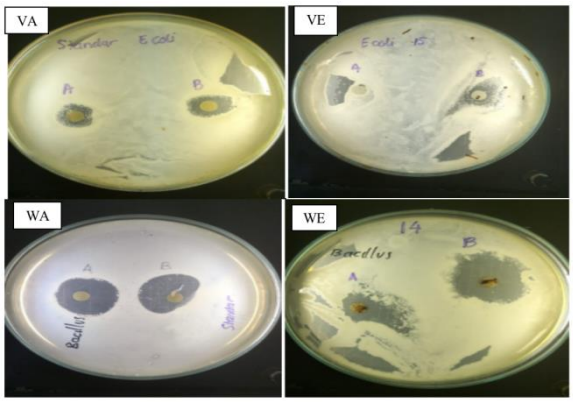

Fig.3. The distance across which the standard drug amoxicillin and the ethanolic extract of RNSB inhibited the growth of *E. coli* (VA and VE) and *B. subtilis* (WA and WE)

4. Conclusion

This study found that the ethanolic extract of RNSB exhibited potent cytotoxic action, with an LC50 value of 186.59 ppm. Moreover, the stem bark extract shows exceptionally potent anti-inflammatory action, with an IC50 value of 3.00 ± 0.009 mg/L. The extract is ineffective against E. coli and B. subtilis. These findings suggest that the stem bark of RNSB has the potential to act as anti-inflammatory and anti-cancer agents. Future studies for RNSB can be carried out through further tests both in vitro and in vivo related to its anticancer and anti-inflammatory activities. In addition, isolation of active compounds and metabolomic studies regarding these activities can be carried out.

5. Conflict of interest

The authors have said that they don't have any other interests that could be seen as competing with this study.

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