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RESEARCH ARTICLE

ANTIBACTERIAL AND PHYTOCHEMICAL SCREENING OF *PIMPINELLA ANISUM* THROUGH OPTIMIZED EXTRACTION PROCEDURE

***Zubaida Marufee Islam, Kashmery Khan, Shagoofa Rakhshanda, Rabab Mahdi and Iftekhar Mahmud Chowdhury**

Department of Mathematics and Natural Sciences, Biotechnology Program, BRAC University, Dhaka, Bangladesh

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ABSTRACT

Since synthetic drugs failed to work on microbial resistance we focused on antimicrobial properties of naturally occurring *Pimpinella anisum*, which may help in the search for newer and less expensive antibiotics. Antibacterial effects of ethanol and methanol extracts taken of second, fifth and seventh days, along with aqueous extracts of aniseed were observed on nine bacteria by disc diffusion method. Aniseed extracts of different days showed positive antibacterial effects on only three bacteria, named, *Bacillus cereus, Bacillus subtilis* and *Streptococcus pneumonae*. The methanol extract from the 5th day showed the highest positive result against *B. cereus* whereas the zone of inhibition for *B. subtilis* was maximum for the 7th day of ethanol extracts. Additionally, the aqueous extract of aniseed showed unremarkable result against *S. pneumonae*. Again, the activity index of methanol extract of day five was the highest against *Bacillus subtilis* indicating high sensitivity to the extract. The phytochemical analysis of the aqueous extract revealed the presence of alkaloids, flavonoids, saponins, tannins, terpenoids, phenolic compounds and cardiac glycosides. As extraction procedure is optimized through this examination, it is expected that the findings of this study will stimulate researchers to design clinical trials that may lead to the development of less expensive antimicrobial agents.

INTRODUCTION

For years now, antibiotic resistance is a vital challenge for the world. It affects not only the pharmaceutical industry, but also the food and beverage industries. As such, it has become one of the main concerns of treating the ever increasing variety of infective diseases in human. Many bacteria are getting resistant to more than a single antibiotic. Such resistant pathogens are hard to treat (Gilchrist, 2007). These pathogens are also called multi-drug resistant bacteria. They may develop due to the unintentional misuse and/or overuse of antibiotics (McEachran, 2015). As a result, alternative sources of more natural antibiotics, in the form of medicinal plants, are being researched these days. Many medicinal plants possess natural antimicrobial compounds. The extracts of such plants have the potential to be used as new agents that are effective against many infections (Wendakoon, 2012). Plants have a wide variety of secondary metabolites such as tannins, alkaloids and flavonoids, which have been found to have antimicrobial properties *in vitro* (Khan, 2009).

****Corresponding author: Zubaida Marufee Islam***
Department of Mathematics and Natural Sciences, Biotechnology Program, BRAC University, Dhaka, Bangladesh

In another way, medicinal plants are safer, more readily available, and cause much less side effects (Hoque, 2011). As a result, assessment of the activities of medicinal plants claimed for having antimicrobial properties is getting interest nowadays. *Pimpinella anisum*, an annual grassy herb which is also known as aniseed has been experimented with positive antibacterial activity against some common microorganisms. The plant falls under the family of Umbelliferae. It is one such plant that has many therapeutic properties. It helps in relieving gastrointestinal spasms and it has carminative properties (Shojaii and Fard, 2012). Moreover, extracts of the aniseeds are used as medicine for their diuretic and laxative effect, expectorant and anti-spasmodic action, and their ability to ease gastric pain and flatulence (Kreydiyyeh *et al.*, 2003). Additionally, the production of milk in lactating women is also observed to increase after the consumption of aniseed. It also reduces the gastrointestinal problems of their children (Shojaii, 2012). Due to all these benefits, it is one of the oldest spices used in traditional medicine. It has also been reported that aniseed is a potent anti-peroxidative and anti-diabetic agent and thereby, possesses a vast spectrum of applications and exploitations in the food and drug industry (Shobha, 2013). Nevertheless, the antibacterial properties of aniseed are not yet fully understood, and so are still being researched. Also, diversified results were observed where various extraction solvents were used to extract aniseed for evaluating antimicrobial activity. Likewise, the antibacterial activities of the aqueous, methanol, acetone and petroleum ether extracts of *Pimpinella anisum* fruits were tested against 4 pathogenic bacteria (*Staphylococcus aureus, Streptococcus pyogenes, Escherchia coli, and Klebsiella pneumoniae*) by disc diffusion method (Akhtar, 2008), and the findings suggest that only aqueous and methanol extracts exhibited fair antibacterial activity against all of the test bacteria. The aqueous extract was found to be more effective than the methanolic extract. Acetone and petroleum ether extracts were incapable of inhibiting the growth of the organisms that were tested (Shojaii and Fard, 2012). Antimicrobial effects of water and ethanolic extracts of aniseed were studied by (Gulcin, 2003), against 10 bacterial species, and *Candida albican.* In another experiment, methanolic extracts of aniseed was tested for its antifungal activity against four dermatophyte species and one saprophyte fungus. In a study (Al-Bayati *et al*., 2008), synergic antibacterial activity of essential oil and methanol extracts of *Thymus vulgaris* and *Pimpinella anisum* was tested against 9 pathogenic bacteria. Positive results were shown by most of the pathogens that were tested. This was also done by measuring the zone of inhibition. The antibacterial potential of aqueous extracts of a few spices, including aniseed was tested in a study (Chaudhry, 2006), against 176 bacterial isolates.

As in the common households of many Asian countries, the people usually eat aniseed in raw form or is used as a spice and cooked. On that context of view, it may be the present concern of research on aniseed where antimicrobial effects rely on the extraction process. Moreover, Extraction duration may affect the ultimate findings of antibacterial activity of aniseed. Specific investigation regarding the timeframe of extraction using different solvents is missing in the previous scientific literatures. Therefore, in the present study, the antibacterial activity has been conducted against *Escherichia coli*, *Salmonella typhii*, *Staphylococcus aureus*, *Shigella flexinera*, *Streptococcus pneumonae*, *Klebsiella sp.*, *Bacillus cereus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*. The aim of the study was to compare antimicrobial activity of aniseed extracted from ethanolic, methanolic, and aqueous treatment. Extraction duration was also optimized to obtain better outcomes in terms of antimicrobial activity against selected nine bacterial strains.

MATERIALS AND METHODS

Collection and processing

The aniseeds were bought from the local store and sundried for about 3 to 4 days. These were then processed through a grinder to turn the seeds into powder form.

Extraction

Ethanolic, methanolic and aqueous extracts were made separately. For the preparation of ethanolic extract 20 gm of the powder was dissolved in 100ml of ethanol, for methanolic extract 100 gm of powder was taken into 500 ml of methanol, and finally for aqueous extract 50 gm of aniseed powder was added to 500 ml of water. For methanol and ethanol, extracts of day 2, 5 and 7 were collected and for water, extract after one hour of mixing was collected. The extracts were filtered using Whatman No. 1 filter paper and concentrated at temperature 80 , 65 and 97 C for ethanol, methanol and water respectively.

Preparation of nutrient agar (NA) plates

Preparation of nutrient agar media was done by adding 28 gm of nutrient agar powder in 1000 ml of distilled water. Keeping this proportion constant, the amount of nutrient agar required was prepared when required. For each medium sized plate 20 ml of agar is needed. This nutrient agar medium is then sterilized by moist heat sterilization method using autoclave at temperature of 120°C at 15 lb pressure maintained for 15 min. , the autoclaved nutrient agar solution was quickly but cautiously poured into previously labeled petri-dishes. This was then kept in the refrigerator to solidify. After that 0.5% McFarland Standard Solution and 0.9% saline solution were prepared.

Sub-culturing of microbes

The stock cultures of the nine microorganisms were taken. To subculture, these were streaked on to the NA plates inside a laminar air flow chamber. For each organism, the plates were taken inside the chamber and then a loop was burned till red hot over a bunsen burner flame. After cooling the loop, a loopful of microbes were taken from the stock culture and streaked onto a properly labeled NA plate. This was then incubated at 37°C temperature for 24 hours before use.

Antimicrobial tests

For conducting antimicrobial tests, agar (or well) diffusion method was used. The microbes from the 24 hours incubated subculture were taken and a bacterial suspension was made. After burning the loop till red hot, a loopful of bacteria was taken and suspended into the saline solution in the test tubes. This was then vortexed for homogenous mixing. Then the tube was visually compared to the McFarland standard solution by holding both of the tubes against a dark background. The turbidity of the suspension was adjusted to match that of the McFarland solution. When the suspension was less turbid more bacteria were added and when it was more turbid more saline solution was added. An autoclaved cotton bud was then taken and dipped into the bacterial suspension. This was done to do lawn culture on properly labeled NA agar plates.

This gives a uniform growth of bacteria. After making the lawn culture, holes were made on the media with the back of an autoclaved micropipette tip. The holes were marked and accordingly the samples of aniseed extracts were poured into the holes with the help of separate autoclaved micropipettes, taking care that a positive control in the form of an appropriate antimicrobial disc is included in each plate. This was then incubated at 37°C temperature for 24 hours, at the end of which the presence of a clear zone around the hole indicated a positive result for antimicrobial tests. This process was followed separately for the ethanol, methanol, and aqueous extracts for each of the nine microorganisms. The activity index (AI) values of the different extracts of aniseed were calculated for the microbes against which positive results were seen. The following formula was used to calculate the AI value:

Activity Index (AI) = zone of inhibition of extracts/ zone of inhibition of the antibiotics

The zone of inhibition was measured in millimeter (mm) with the help of a scale. The width of the clear zone around the antibiotic disc and the well was measured thrice and the average of the three values was noted down.

Phytochemical Tests

Eight different types of biochemical assays were done with aqueous extract of aniseed. These were for tannins, saponins, terpenoids, flavonoids, cardiac glycosides, alkaloids, phenolic compounds and steroids. Around 10 gm of the powdered sample was taken in a beaker along with 100 ml of distilled water. This was boiled for about 10 minutes. The solution was filtered while still hot. Then the filtrate was left to cool down. This was then used to conduct further tests.

Test for tannins

Five to six drops of 10% of ferric chloride is added to 1 ml of the filtrate that was previously diluted with 5 ml of distilled water. When there is a formation of bluish-black or brownish-green precipitate, it indicates positive results for the presence of tannins.

Tests for saponins

To 2.5 ml of filtrate 10ml with distilled water was added to dilute it, and shaken vigorously for 2 minutes. When frothing is observed, it indicates presence of saponins in the filterate. Tests for terpenoids: To 5 ml of extracts 2ml of chloroform was mixed. Then 3 ml of concentrated sulfuric acid was added to form a layer. If reddish brown precipitates are observed at the interface between the two layers, it implies a positive result for the presence of terpenoids.

Tests for flavonoids

To 1 ml of extract, a few drops of 20% sodium hydroxide solution were added. A change of color to yellow indicates a positive result. To reconfirm the test, acid was added and the solution turned back to its original color.

Tests for cardiac glycoside

To 5 ml of extracts 2ml of glacial acid (that contained 1 drop of ferric chloride solution) was added. Then 1 ml of sulphuric acid was added slowly down the side of the test tube. The presence of a brown ring at the interface indicates the deoxysugar characteristics of cardiac glycoside. There may also be a presence of violet ring below the ring while in the acetic acid layer; a greenish ring may be formed.

Tests for alkaloids

To 0.5 ml of the extract, a few drops of picric acid were added down the side of the test tube. A creamy or white precipitate indicates a positive result.

Tests for phenolic compounds

To 5 ml of extract, 5% of ferric chloride was added. If the solution turns to dark green color, it indicates a positive result. Tests for steroids: To 2 ml of extract, 2 ml of chloroform and 2 ml of sulphuric acid was added slowly down the side of the wall of the test tube. Red color produced in the lower chloroform layer indicates a positive result. These methods were followed to check on the antimicrobial and phytochemical properties of aniseed.

RESULTS

Antimicrobial Susceptibility Testing

For the test of antimicrobial properties of ethanolic, methanolic and aqueous extract of aniseed, nine different microbes were used. Amongst these nine microbes, the ethanolic, methanolic and aqueous extract of aniseed showed remarkable positive results against only three (Table 1). The rest either gave negative or nondescriptive results. Different combinations of extracts were put on petridishes to compare the results. Three replicates were made for better accuracy. Antimicrobial discs were used as positive controls. The antibacterials used were ampicilin (for *S. typhii* and *S. aureus*), gentamycin (for *E.* coli and *P. auriginosa*), tetracycline (for *B. cereus*), kanamycin (for *B.subtilis* and *Klebsiella* spp.), chloramphenicol (for *S. pneumonae*) and nitrofurantoin (for *S. flexneri*). The three microbes against which the ethanolic, methanolic and aqueous extract of aniseed showed positive results were *B. cereus*, *B.subtilis* and *S. pneumonae*. The zone of inhibition of chloramphenicol against *S. pneumonae* was the greatest, while that of kanamycin was the least.

Table 1. Positive antimicrobial effects (average zone of inhibition) produced by methanol, ethanol and aqueous extract of aniseed, and that in positive controls

Extraction condition	Day of Extraction	Zone of inhibition (mm)		
		Bacillus cereus	*Bacillus subtilis*	*Streptococcus pneumonae*
Methanol extraction	2^{nd} day	19.03±1.53	18.77± 0.55	12.07±1.39
	5^{th} day	20.13±0.32	19.27± 1.2	14.5±0.65
	7^{th} day	18.43±1.15	15.63 ±1.16	14.87±0.96
	Positive controls	28.61±0.91	23.44± 0.5	31.67±0.88
Ethanol extractiom	2^{nd} day	14.90±0.36	14.01±0.7	12.17±0.12
	5^{th} day	14.52±0.95	12.19±0.9	13.00±0.7
	7^{th} day	14.78±0.94	16.08±1.36	14.44±1.17
	Positive controls	28.20±1.3	23.80±1.47	31.39±1.67
Aqueous extraction	2^{nd} day	11.05±0.69	10.83±1.18	-
	Positive controls	29.56±0.91	24.83±1	-

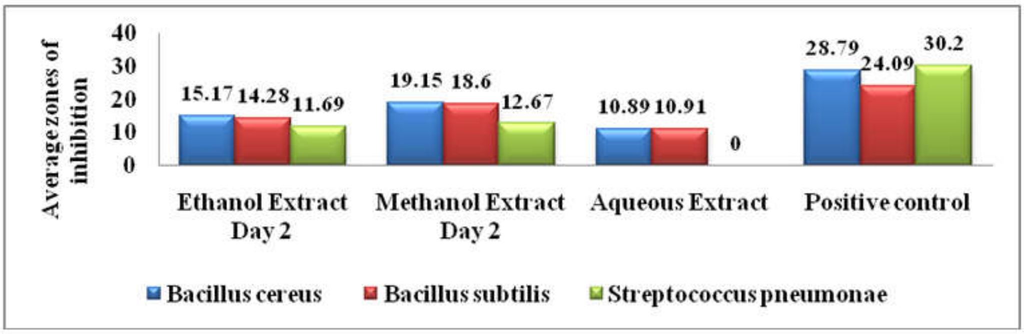
The results represent the mean± SD of values obtained from three independent experiments

In terms of methanolic extraction, the highest and lowest positive results were found against *B. cereus* and *S. pneumonae* respectively. It is also observed that the ethanol extract showed the highest positive result against *B. subtilis*. On the other hand, the lowest positive result was seen against *S. pneumonae*. However, *the* aqueous extract of aniseed had no antibacterial effect on *S. pneumonae*. Against *B. cereus* and *B. subtilis*, it had very little effect.

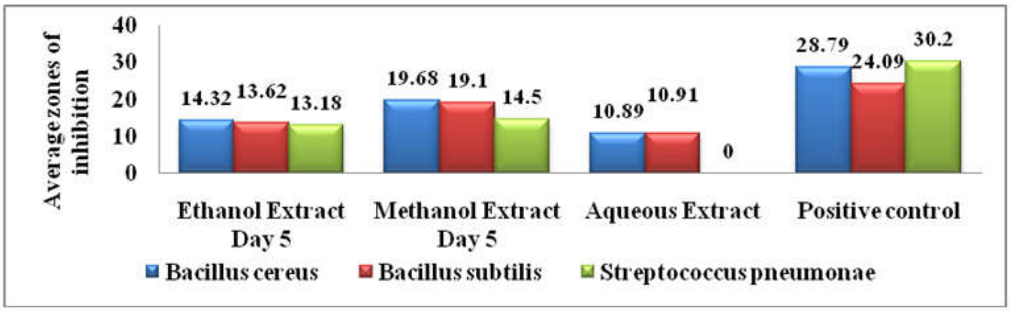
inhibitory effect by showing an inhibition zone of 20.13 mm against *Bacillus cereus* (Table 1). Furthermore, another inhibition zone of 19.27 mm was seen against *Bacillus subtilis* by methanol extracts which was a greater value of inhibition compared to ethanol extracts.

Table 2. Positive phytochemical assay of aniseed in aqueous extract

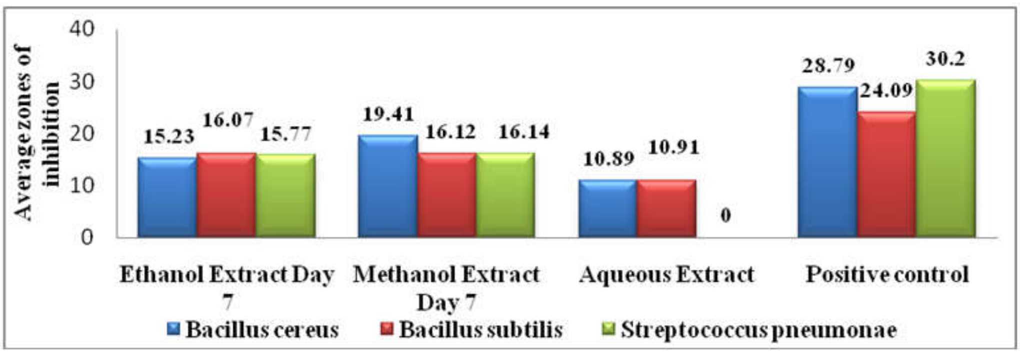
Sl. No	Test	Interference	Result
1	Tannin	Bluish-black or brownish-green precipitate	Present
2	Saponin	Frothing	Present
3	Terpenoid	Reddish brown precipitate	Present
4	Flavonoid	Appearance of yellow color	Present
5	Cardiac Glycoside	Appearance greenish and violet ring	Present
6	Alkaloids	Creamy-white precipitate	Present
7	Phenolics	Appearance of dark green color	Present
8	Steroid	Appearance of red colored product	Present



Graph 1. Average zones of inhibition produced by ethanol, methanol from 2nd day of extraction, along with the aqueous extracts of aniseed and the positive controls



Graph 2. Average zones of inhibition produced by ethanol, methanol from 5th day of extraction, along with the aqueous extracts of aniseed and the positive controls



Graph 3. Average zones of inhibition produced by ethanol, methanol from 7th day of extraction, along with the aqueous extracts of aniseed and the positive controls

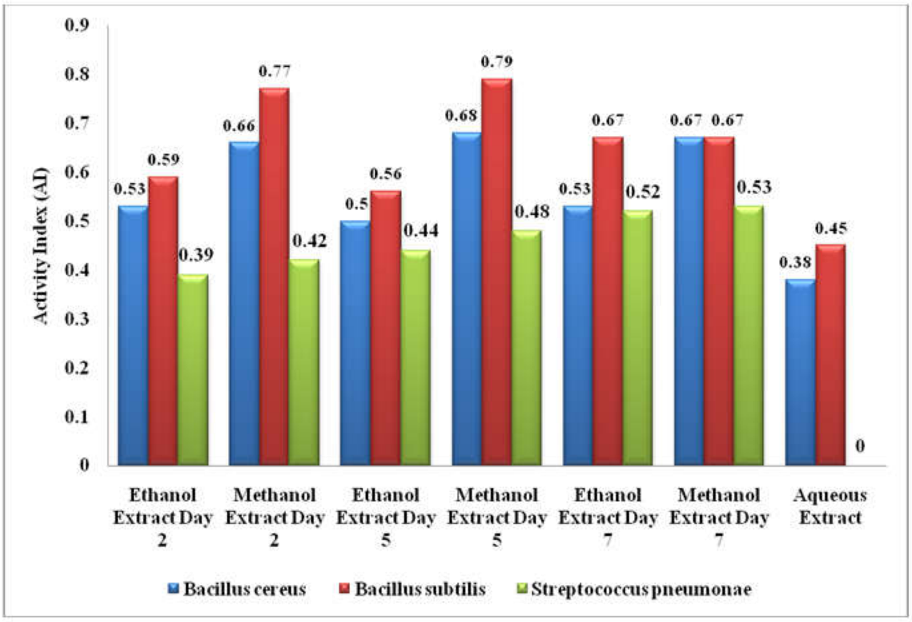
Comparison of antibacterial effects on the basis of using different solvents for extraction: Among three types of extraction solvent, methanol extract of aniseed gave maximum

Conversely, the lowest inhibition of 10.83 against *Bacillus subtilis* was observed from aqueous extraction.

***Comparison of antibacterial effects in different extraction duration*:** Antibacterial effect of 2nd, 5th and 7th days of extracts of ethanol and methanol along with the aqueous extract of aniseed shows that the average zone of inhibition for the three microbes – *B. cereus*, *B. subtilis*, and *S. pneumonae* – was maximum for methanol extract. Among all the 2nd day extractions, methanol extracts found to give the peak inhibitory effect against *B. cereus* (Graph 1). Similar results were observed for the 5th and 7th days of extracts to show antibacterial activity. In case of ethanol extraction, 7th day extracts showed the greater zone of inhibition compared to 2nd and 5th days of extracts against *B. subtilis* (Graph 2 & 3). Phytochemical analysis of aqueous extract of *Pipinella anisum* showed the presence of all eight organic chemicals (Table 2).

DISCUSSION

A study at Maharashtra Animal and Fishery Sciences University revealed antimicrobial effects after 48 hours of mixing aniseed powder with 50% methanol, and with distilled water. The results showed that the aqueous extract was slightly more effective than the methanol extract against all bacteria that was tested using the disc diffusion method. In the present study, the study of the zone of inhibition for the three microbes – *B. cereus*, *B. subtilis*, and *S. pneumonae* – was maximum for the methanol extract from the 5th day of extraction. The aqueous extract of aniseed was not as effective as the ethanol or methanol extracts for any of the three microbes. Another study (Gulcin, 2003) showed that the aqueous extract was made by mixing the aniseed sample with boiling water for only 15 minutes.



Graph 4. Activity Index of the ethanol, methanol and aqueous extracts of aniseed that showed positive antibacterial effects

***Comparison of Activity Index*:** The activity index values of all three types of extracts were calculated using the following formula:

Activity Index (AI) = zone of inhibition of extracts/ zone of inhibition of the antibiotics

The AI value for *B. subtilis* was also maximum for methanol extract from the 5th day. The activity index value for *S. pneumonae* was maximum for methanol extract from the 7th day. The aqueous extract of aniseed showed no value for activity index against *S. pneumonae*, as a consequence of the previous inconsequential antibacterial result against *S. pneumonae* (Graph 4).

Phytochemical analysis

Phytochemical analysis of aqueous extract of *Pipinella anisum* showed the presence of all eight organic chemicals (Table 2), such as, the presence of alkaloids, flavonoids, saponins, tannins, terpenoids, phenolic compounds and cardiac glycosides which could be responsible for the observed antimicrobial properties.

Both the ethanol and aqueous extracts showed strong antibacterial effect against *Staphylococcus aureus*. But, in the current research work, no antibacterial effect was found against *Staphylococcus aureus* for any of the three extracts that were tested. This differences in results may include slight variation in the aniseed grown in the different soil type, the difference in environmental and/or experimental conditions, genotype, and the concentrations of the alcohols used, all of which may have an impact on the extract and its composition. In another study, aniseed sample was mixed with boiled distilled water and left for an hour while alternatively stirring the mixture. The aqueous extract of aniseed had no effect on any of the tested microbes – *E. coli*, *S. typhii* and *Candida albicans* (Mahmood, 2010). The same study by (Gulcin, 2003), also found that the aqueous extract of aniseed showed no antibacterial effect against *P. auriginosa* and *E. coli.* In the test of synergic antibacterial activity of methanol extracts of *Pimpinella anisum* against nine pathogenic bacteria, one of the largest zones of inhibition was observed against *Bacillus cereus* (Al-Bayati, 2008). These results were in coherence with this study, since no results were observed against either of the two microbes, *E. coli* and *S. typhii* for any of the three types of extracts. Although the ethanol and methanol extracts of aniseed from different days of extraction showed good results against *Bacillus cereus*, *B. subtilis* and *S. pneumonae*, the aqueous extracts of aniseed was not as effective on any of these organisms. But rather, it is usually eaten in mixture with water. The *in vitro* antimicrobial effect may reflect that aniseed extract may not be as effective against any or all of these organisms *in vivo*. Another finding of the study is the activity index (AI) values of aniseed from different extraction methods. The AI values are used to find the potential of antimicrobial activity of an extract that is quantitatively compared to the respective standard antibiotics. High AI values imply that the extracts have a good activity against the bacteria in comparison with the standard antibiotics (Sridhar, 2014). In another study (Awan, 2013), the significant use of chloroformic and isoamyl alcohol extracts of a few selected medicinal plants with standard antibiotics was calculated through activity index.

The results from this research suggested that the growth of bacterial pathogens was inhibited by the crude extract of the medicinal plants. The results obtained through activity index were consistent with another study (Shekhawat, 2010). The activity index value of *Justicia neesii* was calculated (Sridhar, 2014). It was observed that the plant extract showed higher AI values against Gram negative bacteria implying that the extracts have good activity against the Gram negative bacteria compared to the standard. In the current experiment, it was found that *B. subtilis* has the highest AI value and the overall AI value of *B. subtilis* is higher than that of *B. cereus*. The AI values of *S. pneumonae* were lower than the AI value of the other two microbes. However, it was also found that on an average, the zone of inhibition against *B. cereus* was much more than that against *B. subtilis*. This is due to the fact that the zone of inhibition of tetracyclin against *B. cereus* was greater than that of kanamycin against *B. subtilis*. Hence, in comparison with the antibiotics, the effect of the aniseed extracts against *B. cereus* was lower than that against *B. subtilis*.

The results from present research work imply that *Pimpinella anisum* extracts have a few antibacterial properties. The different method and day of extraction had a considerable antibacterial effect on a few of the tested organisms. This may be due to the presence of some of its chemical components. The activity of these components may depend on their solubility in different solvents, giving rise to the different results for ethanol, methanol and aqueous extracts. Substituting the commercial antibiotics with extracts of *Pimpinella anisum* or its combination with extracts from other spices with similar antibacterial properties would have potential benefits on human. Medicinal values of many spices lie in the presence of chemical substances that have a definite physiological action on the human body. Different phytochemicals have been found to possess different activities. For example, alkaloids protect against chronic diseases and saponins protect against hypercholesterolemia and also possess antibiotic properties. In a study conducted at Andhra University, various extracts from some spices, including aniseed were screened for the presence of alkaloids, flavonoids, steroids, saponins, tannins and triterpenoid (Harsha, 2013). In a different study (Alkuraishy, 2012), phytochemical analysis of aniseed extracts showed the presence of tannin, Saponins, terpenoids, phenolic agents, flavonoids and alkaloids. The results of this study are in conformity with these studies as it showed positive results for the presence of all of the phytochemical properties, saponins, tannins, terpenoids, flavonoids, cardiac glycoside, alkaloids and phenolics. Considering the overall findings of antimicrobial effects of aniseed extracts in this study, it can be stated that further intensive empirical in-vitro investigation leading to clinical trial may be undertaken.

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