PHYTOCHEMICAL SCREENING AND ANTIBACTERIAL EFFICACY OF BLACK CUMIN (*NIGELLA SATIVA* L.) SEEDS

ABID MEHMOOD[1*], KHALID NAVEED[1], NOMANIA JADOON[1], QASIM AYUB[2], MEHAK HUSSAIN[3] AND MUHAMMAD HASSAAN[2]

[1]*Department of Agronomy, The University of Haripur*
[2]*Department of Horticulture, The University of Haripur*
[3]*Department of Microbiology, The University of Haripur*
Corresponding author email: abidawan1990@gmail.com

خلاصہ

موجودہ دور میں وہ پودے جو بطور ادویات کے منبع کے طور پر استعمال کیا جاتا ہے۔اس کا استعمال بہت ساری بیماریوں کے علاج میں کیا جارہا ہے اور بہت سی بیماریوں کے محفوظ انتخاب کے طور پر استعمال ہو رہا ہے۔دواؤں کے دوسرے پودوں کے ساتھ، کلونجی روایتی دواؤں کی اطلاق کی وجہ سے سب سے زیادہ مشہور ہے۔اس مطالعے کا بنیادی مقصد بیکٹیریا کے گرام مثبت اور منفی تناؤ کے خلاف اس کے پوٹینشل کو تلاش کرنے کے لئے کلونجی کی فائیٹو کیمیکل اسکریننگ کرنا تھا. گرام مثبت اور منفی بیکٹیریا کے خلاف اینٹی مائکروبیل سرگرمی کی جانچ کی گئی تھی کلونجی کے میتھانولک اور آبی عرقوں کا استعمال کرکے۔دونوں نچوڑوں کی پلمونری فائٹوکیمیکل تشخیص نے فلاوونائڈز، الکلائڈ، فینول اور ٹینینز کی موجودگی کی تصدیق کردی۔کلونجی نچوڑنے منتخب مائکروبس کی نشوونما کو روک کر پاور اینٹی مائکروبیل سرگرمی کی بھی نمائش کی۔۔مندرجہ بالا تجربے سے یہ ثابت ہوئی ہے کہ کلونجی نباتاتی کمیائی تجزوبے میں مختلف نباتاتی مرکبات کی موجودگی کی تصدیق ہوئی۔جس سے ثابت ہوا کہ کلونجی کے بیج مختلف بیماریوں کے علاج کے لئے استعمال کے جاسکتے ہیں.

Abstract

The use of medicinal plants against the treatment of many diseases is now widely under consideration and is being used as a secure choice for many diseases. Along with other medicinal plants, black cumin is most popular due to its traditional medicinal application. The main aim of this study was to perform phytochemical screening of black cumin in order to explore its potential against gram positive and negative strains of bacteria. Qualitative analysis of phytochemicals like tannins, terpenoids, anthocyanins, flavanoids, steroids, coumarins, leucoanthocyanins, cardiac glycosides, saponins and diterpenes were performed. Antimicrobial activity against gram positive and negative bacteria was investigated by using methanolic and aqueous extracts of black cumin. Phytochemical evaluation of both extracts confirmed the occurrence of flavonoids, alkaloid, phenol and tannins. Black cumin extract also exhibited the power as anti-microbial activity by inhibiting the growth of selected microbes. This study confirms the occurrence of vital phytochemicals in the extracts of black cumin seeds and thus can be concluded that seeds of black cumin can be used for the treatment of various diseases.
Keywords: Black cumin, *Nigella sativa*, Phytochemicals, Antibacterial activity, *Bacillus subtilis*

Introduction

Spices are consumed and appreciated for unique flavor and aroma and entered the agricultural activity of humans around 6000 BC though their presence was recorded in Neolithic era or even earlier (Mehmood *et al*., 2018). Among the spices, seed spices are an important group that consists of around 20 spices. Black cumin (*Nigella sativa* L.) is considered as a *miraculous spice* having very important medicinal values apart from its intrinsic flavor (Naz, 2011). Historical records show that plants are being used from thousands of years for treatments of many diseases. The plant materials are either used directed or administered in the form of teas, powder etc. (Shahzad *et al*., 2020). The development and production of drugs by using medicinal plants started by isolating primary compounds directly from plants, these drugs include digitoxin, quinine, cocaine, and codeine, some are still in use for different purposes (Agarwal *et al*., 2019). Black cumin also known as Kalonji locally, is one of the most widely examined plant possessing wide range of naturally occurring compounds (Mehmood *et al*., 2018). It is a field crop that belongs to *Ranunculaceae*, the butter cup family. In Islam, black cumin is considered as the best healing medicine available. Hence, it is appropriately known as *seed of blessing* (*habbatul barakah*) (Srivastava, 2014). The medicinal value of black cumin is of immense importance and numerous workers appreciated its unique, varied and powerful pharmacological traits. The medicinal importance of the herb is widely reported (Gilani *et al*., 2004). Hence to validate the traditional medicinal importance of black cumin, chemical analysis of the seeds is of greatest interest, so that its potential in the cure of many diseases can be examined and to find its active principles by characterization and isolation of its

components. The phytochemical screening can help in confirmation and presence of already explored phytochemicals but can lead to the discovery of new active compounds.

Materials and Methods

The study was conducted in Agronomy Laboratory, The University of Haripur during 2020. Seeds of black cumin variety NARC-1 Kalonji were taken from Agricultural Research Farm, The University of Haripur. The seeds were ground into fine powdered form by using electric grinder and kept in sealed glass bottle at room temperature for further use. About 50g ground seeds were placed in a round bottom flask and 375 ml methanol along with 375 ml distilled water was mixed together. The mixture was kept in dark for 72 h with frequent stirring. The filtrate was isolated from the mixture by method of filtration, using Whatman filter paper. In order to obtain the dry extract, the filtrate was heated at 50-60°C by using a rotator evaporator, the heating was carried out till the evaporation of filtrate. The dry extracts were then collected from the round bottom flask and kept at 4°C for further experimental studies (Harborne, 1973).

Phytochemical Analysis

Detection of phytochemicals like flavonoids, alkaloids, tannins and saponins was performed on the extracts by the method described by Sofowora, (2008).

Test for Alkaloids

15 mg of both extracts were taken separately and stirred with 6 ml of 1% dilute hydrochloric acid and placed on the water bath for 5 minutes and then filtered. The filtrates were divided into three equal parts for further analysis.

(a) Dragendorff's Test

01 mL Potassium Bismuth Iodide solution (Dragendorff's reagent) was thoroughly mixed in the filtrates. The appearance of an orange red precipitate indicated the presence of alkaloids.

(b) Mayer's test

Potassium Mercuric Iodide solution (Mayer's Reagent 1 ml) was poured in to one portion of filtrate and homogenized thoroughly. The appearance of cream color precipitate showed the occurrence of alkaloids.

(c) Wagner's test

100 ml solution of (1.25g) iodine and (2g) of potassium iodide was prepared and few drops of the solution was mixed in the filtrate, the appearance of brown color precipitate indicated the presence of alkaloids.

Test for Steroids

0.1g of crude extract was dissolved in 10 ml chloroform and 1 ml of conc. H_2SO_4 was then added to the mixture. The presence of steroids was then determined by the appearance of yellow with green fluorescent colored layer over sulphuric acid.

Test for Flavonoids

Diluted NaOH (sol.) was added in 0.5ml crude extracts of plant. A dark yellow color appeared in the plants extract, that disappeared when dil. H_2SO_4 acid was added to the mixture, which confirmed the presence of flavonoids (Ahmad and Beg, 2001).

Test for Terpenoids

(a) Salkowski test

Mixture of plant extract (100mg) + chloroform (2ml) +conc. H_2SO_4(2ml) was made and thoroughly shaken in a test tube, the presence of terpenoids was noted by the appearance of reddish-brown color.

(b) Liebermann-Burchard test

100 mg extract was shaken in test tube with chloroform, and then acetic anhydride drops were mixed in the mixture and boiled in hot water bath followed by rapid cooling with chilled water. 2ml of conc. H_2SO_4 was then added to the mixture. The formation of brownish colored ring at the junction of two layers and transformation of upper layer into green color indicated the presence of steroids, finally the formation of dark red color in lower layer showed the presence of triterpenoids.

Test for Tannins Extract

100mg extract was separately stirred with distilled water (5 mL) and then filtered. A few drops of 5% ferric chloride solution then added. Blue-green color precipitate was taken as positive result for the presence of tannins.

Test for Saponins

50 mg crude extract was mixed with 10ml distilled water in a test tube, which is then hand shaken for 15mins. The appearance of foamic layer on the top of mixture indicated the presence of saponins.

Tests for Glycosides

(a) Anthraquinone Glycoside (Borntrager's test)

01 mL of dried filtrate was mixed with 5% H_2SO_4 and the solution was boiled in hot water bath. After boiling equal amount of chloroform was added to the solution followed by vigorous shaking of the solution, and kept in stand for 5 min until the formation of bi-layers of chloroform. After that half volume of chloroform and diluted ammonia was added, that formed rose pink red layer of ammonia, indicating the presence of anthraquinone glycosides.

(b) Cardiac glycoside (Keller-Killiani test)

1mL dried filtrate of cumin extract was shaken with 5mL of distilled water, then 2mL of glacial acid was added to the mixture along with few drops of ferric chloride ($FeCl_3$) and 1mL H_2SO_4. The appearance of brown ring indicates the presence of cardiac glycoside.

Anthocyanin

The detection of Anthocyanin was done by the addition of 2mL HCl and HNO_3 to the aqueous solution of dried filtrate, the formation of blue violet color showed the presence of Anthocyanin.

Coumarin

Coumarins were detected by mixing 3mL of NaOH (10%) with 2mL of extract. The formation of yellow color showed the presence of coumarins.

Test for Phytosterols

1 mL of chloroform and dried plant extracts were mixed together followed by addition of few drops of H_2SO_4. The formation of brown colored ring showed the presence of steroids.

Test for Polyphenols

2mL of distilled water along with few drops of ferric chloride (10%) was added to the 1mL aqueous plant extract. The blue color formation showed the presence of polyphenols.

Antibacterial study

To identify the anti-bacterial activity of black cumin both gram positive and gram-negative bacteria were utilized. Antibacterial activity of black cumin seeds extract was determined by the procedure described by Nwonuma *et al*., (2020) by using Modified Agar Well Method. Swabbing of nutrient agar plates was performed with the help of sterile cotton swab, by using 24 hours old broth culture of selected bacteria. Afterwards a 0.5 cm well was made by using sterile borer in every plate. 500 µg/mL of plant extract (aqueous and methanol extracts) was filled into each well. Later on, the diffusion of extract into agar was done by placing the NA plates at room temperature for one hour. After complete diffusion of extract, the plates were placed in incubator at 37 °C for 24 hours. The antibacterial activity was then calculated by measuring the diameter of inhibition zone after 48 hours. Amoxillin and gentamycin drugs were used as standard for measuring antibacterial activities.

Results and Discussion

It was revealed that methanolic extract of black cumin contains alkaloids, steroids, flavonoids, tannins, saponins, glycosides, coumarins, phytosterols and polyphenols. Alkaloids, flavonoids, saponins and coumarins showed their higher presence in methanolic extracts. Tannins, and glycosides showed moderate presence while weak presence of steroids, phytosterols and polyphenols was observed. Terpenoids and Anthocyanins were not present in methanolic extracts. Alkaloids are proven to cause the beneficial biological compound for human health and an important part of diet for ages. Recently, it is used as supplements in pharmaceuticals, and in other applications in human life. Moreover, they showed the potential for organic synthesis for searching new semisynthetic and synthetic compounds with possibly better biological activity in controlling and preventing many diseases than parent compounds (Shahzad *et al*., 2018). Water (aqueous) as polar solvent and methanol as solvent of intermediate polarity was used for the extraction of secondary metabolites that are different from one another in polarity and structure. Thus, both of the solvents showed distinct biological properties. During the process of extraction, solvents usually diffuse into plant materials and the compounds of similar polarity solubilize (Owoyemi *et al*., 2017). The composition and quality of secondary metabolites of an extract is affected by the polarity of the solvent. Traditionally, water is used by the healers for the preparation of plant

extracts but organic solvents like methanol gives more consistent antibacterial activity and quality secondary metabolites as compared with aqueous (Mudzengi *et al*., 2017). Aqueous, methanolic, hexane and ethanol were used most commonly as solvents for antibacterial investigations (Abba *et al*., 2020).

Agar well dilution method was used against four strains of bacteria which are; *Pseudomonas syringica*, *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus* sp. *Bacillus subtilis* and *Staphylococcus* were gram positive while *Pseudomonas syringica* and *Escherichia coli* were gram negative. *B. subtilis*, *E. coli* culture in petri-plates was incubated for 24 hours to observe the growth of bacteria into inhibition zone. Afterwards the anti-bacterial activity of both methanolic and aqueous extracts of black cumin seeds was calculated.

The present investigation indicates that methanolic extract of black cumin seeds significantly suppress gram positive bacteria growth as well as gram negative bacteria. Maximum inhibition zone was found in *Pseudomonas syringica* as listed in Table 02.

Bacteria like *E. coli* which is used in this study causes UTI (Urinary Tract Infections), diarrhea, meningitis and sepsis (Bichler *et al*., 2002); (Adegoke *et al.*, 2010). *S. aureus* is responsible for the spread of skin infection, septicaemia and is a major pathogen causing food poisoning and urinary tract infection (Adegoke *et al*., 2008); (Bichler *et al*., 2002). Same results were also observed by earlier researchers, who noted that major portion of medicinal plants have least effectiveness against gram negative as compared to gram positive organisms (Ignacimuthu *et al*., 2010).

It was observed that methanolic seed extract of black cumin exhibited anti-bacterial activity towards all the strains of studied bacteria. The anti-bacterial activity of seed extracts can be due to the presence of Thymol (Had *et al.*, 2016) which possesses anti-bacterial properties (Tayel *et al*., 2018). Tariq *et al*. (2016) reported that thymol causes phenolic toxicity to microorganisms, include enzyme inhibition by the oxidized compounds, possibly through nonspecific interactions with the proteins.

Table 1. Phytochemical Analysis of Black Cumin Seed Extracts

Phytochemicals	**Extracts**	
	Aqueous	**Methanol**
Alkaloids	+	+++
Steroids	-	+
Flavonoids	+++	+++
Terpenoids	-	-
Tannins	++	++
Saponins	+	+++
Cardiac Glycosides	-	++
Anthocyanins	-	-
Coumarins	+++	+++
Phytosterols	+	+
Polyphenols	+	+

+ = Weakly present, ++ = Moderately present, +++ = Highly present, - = Not present

Table 2. Antibacterial activity of aqueous and methanolic extracts of Black Cumin.

Microorganism	**Solvent**	**Diameter of the zone of inhibition (mm)**
Bacillus subtilis	Methanol	17.3 ± 0.8
	Aqueous	12.6 ± 0.3
Staphylococcus	Methanolic	12 ± 1.1
	Aqueous	4 ± 0.5
Pseudomonas syringica	Methanolic	20.6 ± 0.8
	Aqueous	8 ± 0.5
Escherichia coil	Methanolic	13 ± 0.5
	Aqueous	6.6 ± 0.8

Note: Values are expressed as mean ± SE (n = 3)

Conclusion

Over all, the resistance of bacteria towards antibiotics is a matter of great concern for modern medical industries. The result of present investigation reveals the strong anti-bacterial activity of black cumin seeds extracts against studied bacterial pathogens. Furthermore, phytochemicals screening of black cumin seeds confirms the presence of medicinally vital secondary metabolites, which can show antibacterial characteristics. More intensive research in this regard is recommended to further explore the antiviral potential of black cumin towards life threatening viral diseases e.g. HCV, Dengue and Covid-19.

Acknowledgement

The authors are grateful to the Department of Agronomy and Department of Microbiology, The University of Haripur for providing the opportunities to work in their Laboratories.

References

Abba, G.B.A., Mohammed, T.I., Wali, J.M., Adamu, M.M., Mahmud, K.M., Barma, A.F. and Garba, B.A., 2020. Phytochemical characterization and antimicrobial studies on four folklore medicinal plants in Semi-Arid Region of Borno State, Nigeria. *World Journal of Advanced Research and Reviews*, *7*(1), pp.001-006.

Adegoke, A.A. and Komolafe, A.O., 2008. Nasal colonization of school children in Ile-Ife by multiple antibiotic resistant Staphylococcus aureus. *International Journal of Biotechnology and Allied Sciences*, *3*(1), pp.317-322.

Adegoke, A.A., Iberi, P.A., Akinpelu, D.A., Aiyegoro, O.A. and Mboto, C.I., 2010. Studies on phytochemical screening and antimicrobial potentials of Phyllanthus amarus against multiple antibiotic resistant bacteria. *International Journal of Applied Research in Natural Products*, *3*(3), pp.6-12.

Agarwal, G., Carcache, P.J.B., Addo, E.M. and Kinghorn, A.D., 2019. Current status and contemporary approaches to the discovery of antitumor agents from higher plants. *Biotechnology advances*, 38, pp. 1-27.

Ahmad, I. and Beg, A.Z., 2001. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. *Journal of ethnopharmacology*, *74*(2), pp.113-123.

Bichler, K.H., Eipper, E., Naber, K., Braun, V., Zimmermann, R. and Lahme, S., 2002. Urinary infection stones. *International journal of antimicrobial agents*, *19*(6), pp.488-498.

Gilani, A.U.H., Jabeen, Q. and Khan, M.A.U., 2004. A review of medicinal uses and pharmacological activities of *Nigella sativa*. *Pakistan Journal of Biological Sciences*, *7*(4), pp.441-451.

Hadi, M.Y., Mohammed, G.J. and Hameed, I.H., 2016. Analysis of bioactive chemical compounds of Nigella sativa using gas chromatography-mass spectrometry. *Journal of Pharmacognosy and Phytotherapy*, *8*(2), pp.8-24.

Harborne, A.J., 1998. Phytochemical methods a guide to modern techniques of plant analysis. *springer science & business media*.

Harborne, J.B., 1973. *A guide to modern techniques of plant analysis*. Chapman and Hall.

Ignacimuthu, S. and Shanmugam, N., 2010. Antimycobacterial activity of two natural alkaloids, vasicine acetate and 2-acetyl benzylamine, isolated from Indian shrub Adhatoda vasica Ness. leaves. *Journal of biosciences*, *35*(4), pp.565-570.

Mehmood, A., Naveed, K., Azeem, K., Khan, A., Ali, N. and Khan, S.M., 2018. Sowing time and nitrogen application methods impact on production traits of Kalonji (*Nigella sativa* L.). *Pure and Applied Biology (PAB)*, *7*(2), pp.476-485.

Mudzengi, C.P., Murwira, A., Tivapasi, M., Murungweni, C., Burumu, J.V. and Halimani, T., 2017. Antibacterial activity of aqueous and methanol extracts of selected species used in livestock health management. *Pharmaceutical biology*, *55*(1), pp.1054-1060.

Naz, H., 2011. *Nigella sativa*: the miraculous herb. *Pakistan Journal of Biochemistry and Molecular Biology*, *44*(1)**,** pp.44-48.

Nwonuma, C.O., Adelani-Akande, T.A., Osemwegie, O.O., Olaniran, A.F. and Adeyemo, T.A., 2020. Preliminary in vitro antimicrobial potential and phytochemicals study of some medical plants. *F1000Research*, 8(81), p.81-99.

Owoyemi, O.O. and Oladunmoye, M.K., 2017. Phytochemical screening and antibacterial activities of Bidens pilosa L. and Tridax procumbens L. on skin pathogens. *International Journal of Biological and Medical Research*, *8*(1), pp.24-46.

Shahzad, Q., Sammi, S., Mehmood, A., Naveed, K., Azeem, K., Ahmed Ayub, M.H., Hussain, M., Ayub, Q. and Shokat, O., 2020. Phytochemical analysis and antimicrobial activity of adhatoda vasica leaves. *Pure and Applied Biology (PAB)*, *9*(2), pp.1654-1661.

Sofowora, A., 2008. Medicinal Plants and Traditional Medicine in Africa, 289-296.

Srivastava, B., 2014. Medicinal and the rapeutical potential of *Nigella sativa*. *International Journal of Medical and Applied Sciences Research*. *1* (1), pp.32-39.

Tariq, A., Adnan, M., Amber, R., Pan, K., Mussarat, S. and Shinwari, Z.K., 2016. Ethnomedicines and anti-parasitic activities of Pakistani medicinal plants against Plasmodia and Leishmania parasites. *Annals of clinical microbiology and antimicrobials*, *15*(1), pp.52-65.

Tayel, A.A., Shaban, S.M., Moussa, S.H., Elguindy, N.M., Diab, A.M., Mazrou, K.E., Ghanem, R.A. and El-Sabbagh, S.M., 2018. Bioactivity and application of plant seeds' extracts to fight resistant strains of Staphylococcus aureus. *Annals of Agricultural Sciences*, *63*(1), pp.47-53.