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Research Article

In vitro Antibacterial, Antifungal and GC-MS Analysis of seeds of Mustard Brown

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Abstract

In this research work, antibacterial, antifungal and GC-MS analysis was carried out. For this, seeds extract of Mustard brown was prepared from methanol and fractions with solvents *n*-hexane, chloroform, ethyl acetate, and acetone; microbiologically tested against bacteria such as *Rhodococcusspp*, *Bacillus subtilis* and *Escherichia coli* and also against fungi such as *Aspergillus niger*, *Aspergillus flavus* and *Trichoderma harzianum*. Methanol extract and *n*-hexane fraction of methanol extract exhibited maximum antimicrobial inhibition activity. Acetone and *n*-hexane fraction of methanol extract of plant exposed highest antifungal activity. It is concluded that plant is an opulent source of phenols that is responsible for inhibitory influences on bacteria and fungi. Whole results anticipated the plant antibacterial and antifungal potential and are valuable contender for the cure of several kinds of therapeutic illness.

Keywords: Mustard, fraction, extract, antibacterial, antifungal.

1. Introduction

For centuries plants have been castoff as medications for humanoid ailments because they hold constituents of therapeutic standards [1]. The medicinal plants have a traditional usage in the history; some of their biological activities have just been scientifically characterized [2,3]. It acknowledged that plants are chief therapeutic spring to extravagance the contagious ailments in some emerging nations [4]. Humanoid toxicities chiefly relating to microbes are responsible for serious contaminations in stifling and subtropical republics [5]. Curative plants serve as antimicrobials by manufacturing hydrolytic enzymes, proteins, and essential oils as well as by making ph-enolics compounds. Lesion contamination frequently produced by five sorts of microbes such as *Staphylococus aureus*, *Streptococcus pyogens*, *Coryne bacterium spp*; *Escherichia coli*, *Pseudomonas coli* [6]. Consequently, there is an incessant and imperative requirement to determine new antimicrobial composites have assorted biochemical configurations and innovative course of action [7]. There is a wide spread in drugs derived from plants. Naturally occuring antimicrobial medicines can be resultant from various parts of plants i.e. fruitlets, floras, verdures, trunks, and woofs [8]. It has reprted that members of the *Brassicaceae* can inhibit fungal plant pathogens when grown as a green manure crop or added to soil as seed meal or plant residues [9]. Different studies revealed the suppressive effects of mustard tissues as they contain glucosinolates which is produced by mechanical disruption of the plant material, can be enzymatically degraded to form volatile antimicrobial compounds [10]. Mustard brown belongs to a family of *Brassicaceae* and genus *Brasica* [11]. Mustard brown is also known as Indian mustard. It had been stated that mustard brown is an old-fashioned tonic for stiffness, foot aching, back pain inoffensive, aperitif, stimulant and rheumatism [12]. It is reported that seeds of Mustard brown used for the treatment of malignant tumors in China and roots of Mustard brown had been employed as a galactagogue in Africa. Different studies revealed that in Tanganyika, its leaves as well as its flowers was smoldered to produce an aroma which revolting to mosquitoes. It had been assumed towards this plant to be aperients and booster so its volatile oil had been castoff as a counterirritant and intoxicating. Mustard brown has been employed as an antisyphilitic emmenagogue in Javaas well as its leaves was used for the relieve of headache [13]. Reported data shows that people eat the plants leaf for the curement of swelling of bladder in china [14]. Mustard brown is also used in the treatment of inflating aching of ribs, deep abscess, arthritias, menostasis and brainy seepage [15]. Previous studies exhibited that in Yunani as well

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as Ayurveda, seeds of Mustard brown has been used for the curement of skin ailments, viscera diseases as well as infections caused by worms. Brown mustard has a component which is employed in numerous Ayurveda medicinal emollient as this oil is castoff as ointment in the curement of several paralytic maladies of central nervous system [16]. It is reported that oil of Mustard brown has been applied as an antidote in large concentrations in poisoning circumstances [17]. Oil extracted from Mustard brown has established a pronounced interest to be used in phytotherpeutical medicines. Different research work shows that Mustard brown also displays preventing measurements on the development of bacteria as well as fungi that is responsible for food poisoning [18]. Mustard brown plant also exhibits suppressive outcomes on cancer cells [19]. The main objective of present research work was to assess the polarity base extraction of seeds of Brown mustard to evaluate and compare antibacterial as well as antifungal potential of methanolic extract and other polarity base fractions and their GC-MS analysis study.

2. Materials and Methods

Seeds of Brown mustard were collected from the local market of Lahore in June, 2015. The seeds were subsequently confirmed from the taxonomist, Department of Botany, Government college University Faisalabad. All research work was done at organic laboratory in the Department of Chemistry, University of Management and Technology Lahore as well as at NIBGE Institute Faisalabad Pakistan. All chemicals were employed of analytical grade which were procured from BASF Germany as well as Merck. Standard drug used for antimicrobial as well as for antifungal activity are Kanamysin and Terbinafine hydrochloride respectively.

2.1 Extract Preparations

Plant material was cleaned in order to remove dirt and extraneous matter. They were dried afterwards in shade, and cleaned material was crushed in order to get a pulverized powdered form. The plant material (1000g) was washed with cold water, dry in shady place and the dried out taster has been grinded by the use of a blender and passes through 70 mesh sieves. This crushed sample was taken in reagent bottle and about 1.5 liters of methanol was added. It was placed for seven days, with daily shaking of about five minutes. After the seven days, methanol extract was filtered with Whattman filter paper no.1, and filtrate was allowed to evaporate in air while keeping it in shady place. The same procedure was repeated for three times to get maximum amount of extract. The weight of extract was taken to find out percentage yield. Different fractions of methanol extract were taken by using different solvents like *n*-hexane, chloroform, ethyl acetate, acetone to check their antimicrobial activity.

2.2 Fractionations of plant material

A small portion of methanol extract was saved for further use. Methanol extract was further fractioned with *n*-hexane by employing approximately 20 ml of *n*-hexane in methanol extract and mixed with the help of glass spatula until solvent color was altered which indicating that the extraction with *n*-hexane has been completed. Afterward of extreme extraction, *n*-hexane solvent was vaporized in surrounding air to get sticky material and taken in separate bottle which was covered with aluminum foil and saved at cool and dry place for further use. Fractions of chloroform, ethyl acetate and acetone were made as like *n*-hexane fraction obtained.

2.3 Assessment of antibacterial activity

Well method was used to load sample on agar plates on which microbial culture was already spread and antimicrobial activity is determined by measuring the size of inhibition zone on agar petri dish. Antibacterial potential of extract and fractions of Mustard brown was tested in counter to nominated cultured bacteria such as *Rhodococcusspp*, *Bacillus subtilis* and *Escherichia coli* in which two are Gram-positive and one is Gram-negative respectively. Antibacterial potential of extract and fractions of Mustard brown was tested in comparison to standard drug Kanamysin. Sample solution for antimicrobial activity was prepared with concentration about 250mg of each fraction in 1ml of dimethyl sulfoxide (DMSO).

2.3.1 Nutrient Agar Media preparation and configuration:

Weight accurately about 28 grams nutrient agar which was added in distilled water (1000 ml) and mixed by magnificent stir process to dissolve well and to make the solution uniform [20].

2.3.2 Media sterilization process

After that, media suitably persevered with yarn and media (Nutrient agar) was placed in flask and was incubated in Autoclave. Autoclave cover was made immobile and kept temperature at 121^0C and maintains pressure at 15 LD/ sq inch for 15 minutes to sanitize the media. After 15 minute, autoclave was permitted to cool and flask was detached from autoclave and placed the nutrient agar flask in water bath at 50 ^{0}C for further use in research study.

2.3.3 Petri plates preparation

Autoclaved solution about 200 ml was transferred in autoclave. Kept the petri dishes and medium overnight to solidify. In next day to eliminate moistness, lid of petri dishes were heated in Laminar flow burner and then plates were retained for half an hour in freezer. Then bacteria culture of about 105 µl of each was spread by the use of micropipette on seven petri dishes and bacterial cultures were placed on entire medium surface by the use of pasteurized propagator.

2.3.4 Well loading methodology

In this current research work, extracts of Mustard brown are diffused from several crater through hardened agar layer, so that development of incubated microbes are prohibited completely in rounded zone [21]. In this study, by the use of 200µl tip of micropipette, seven wells were prepared in intermediate medium. Centrifuged tasters of methanol extracts and factions of n-hexane, chloroform, acetone as well as DMSO (control) and antibacterial drug, Kanamycin sulfate were transferred in wells and all samples were placed in incubator overnight. The incubator delivers promising hotness for microbe's development and next day reticence area were patterned and calculated in millimeter.

2.4 Assessment of antifungal activity

Antifungal activities of the extracts of Mustard brown seeds were acquired by means of the size of inhibition zones on Vogel mass media substrate [22].

2.4.1 Fungoid cultures preparation

Fungal stains such as *Aspergillus niger*, *Aspergillus flavus*, and *Trichoderma harzianum* were given by the Industrial Biotechnology Division of NIBGE Faisalabad, already cultured in their relevant media substrate.

2.4.2 Vogel media development and configuration

Fungal has been developed on various media and has composition which is Trisodium citrate (2.5g), KH_2PO_4 (5.0g), NH_4NO_3 (2.0g), $(NH_4)_2SO_4$ (4.0g), $MgSO_4.7H_2O$ (0.2g), $CaCl_2.7H_2O$ (0.1g), Peptone (2.0g), Glucose (5.0g), Agar (12.0g), pH~5.5 and Distill water (1000ml).

2.4.3 Sterilization of fungal media

For sterilization, flask containing the solution was properly plugged with cotton and encumbered in the research laboratory autoclave. The cap of autoclave was immobile and protection tap is accustomed to the prerequisite pressure and temperature. Once the requisite temperature (121ºC) and pressure (15 Ib/sq. inch) were gotten then mass media was permitted to sanitize for 15 min and allowed to cool to about 50 centigrade.

2.4.4 Petri plates preparation

Prepared solution as well as washed petri dishes were autoclaved then cool the solution. Approximately 200ml solution was transferred in seven petri dishes. The petri dishes were allowed to stay overnight until the media was gotten hardener and solidify itself. Heat was given to the lid of petri dishes to get rid of vapors and petri dishes were stored in refrigerator for half an hour. Fungiform cultures of about 100 µl were inoculated on all petri dishes by means of micropipettes.

2.4.5 Loading of plates for fungal cultures

The whole process of loading was carried out in laminar flow burner specified for fungal cultures. Before loading plates were labeled carefully. For the preparation of plates about 100 micro liter of strain was spread on the solidified media with the help of spreader. Before using the spreader, the spreader was rinsed with rectified spirit and exposed to the burner. Then well was formed with the help of sterilized tip attached to the pipette man. About 100 µl of sample as well as control and standard solutions of Terbinafine hydrochloride was applied to separate wells on fungal plates.

2.4.6 Growth of fungal cultured plates

After the completion of loading the plates were placed in an incubator for the growth of microorganisms, the temperature of incubator was fixed at 37 centigrade. The purpose of incubation is to provide optimum temperature for fungal growth. After 12-14 hours the growth was appeared. The growth was checked with the help of Venire caliper in mm by measuring the zone of inhibition around the sample well.

2.5 Isolation of Essential Oil:

The whole plant (500 g) dried out then pulverized into powder was hydr-odistilled for 4.5h by means of Cleve-nger style device as pronounced previously by [23,24]. The fraction crop of essential oil of plant was found to

be 0.40%. The essential oil of plant was serened and dehydrated in the presence of Na_2SO_4 then sifted and kept at 4°C till further analysis.

2.6 GC-MS Analysis of Essential Oil:

The tester was examined by means of a GC 6850 network GC system equipped with a 7683B series auto injector and 5973 inert mass selective detector (Agilent Technologies, Willmington, DE, USA). Chemical composites were detached on an HP-5 MS capillary column with a 5% phenyl polysiloxane stationary phase (30.0 m × 0.25 mm, film thickness 0.25 µm). Temperature of oven was planned in a three phase ascent: early temp fixed at 45°C (detained for 5 minutes), continue until temperature reached to 150°C at 10°C per minute, then in second phase, allowed the temperature to be raised by a 5 °C per minute till 280°C and lastly temperature attained 325°C by rising 15°C per minute and temperature was detained for 5 minute at this stage. Flow rate of Helium gas was kept 1.1 mL per minute and pressure is kept 60 Kilo Pascal and its linear velocity to be remained 38.2 cm per second. Ions as well as fragments were examined in scanning manner through 40–550 *m/z*.

2.7 Identification of Compounds:

The recognition of the chemical constituents was constructed on the basis of their retention index (RI) comparison, comparative to an alkane series (C8–C25) that are to be standard. The composites were additionally recognized as well as verified by employing comparison between the MS statistics of analyte composites with the published mass spectral data as well as also along with the NIST 05 Mass Spectral Library [25]. The numerical facts were attained electronically from the area percentage of FID deprived of the usage of any rectification aspects.

2.8 Statistical Investigation:

All investigational exertion was completed with smallest regular of three periods. The statistics was presented with mean ±SD beliefs at 95% confidence interval. Significant differences ($p < 0.05$) were resulted among means.

3. Results and Discussion

3.1 %age yield of extracts:

The yields of different fraction (*n*-hexane, Chloroform, Acetone and Ethyl Acetate) and extract (Methanol) of Mustard brown seed were founded within the range of 0.175g to 0.60 g. Highest crop of yield 0.6g was perceived with methanol, while lowest yield 0.175g with acetone. Figure 1 represented the graphical representation of %age yield which is obtained from the extract of Mustard brown seed and its different fractions. The %age yields of different fractions as well as extract of seed of Mustard brown in different solvent schemes lessening in the subsequent direction, Methanol (0.6) > *n*-hexane (0.5) > Chloroform (0.25) > Ethyl acetate (0.2) > Acetone (0.175)

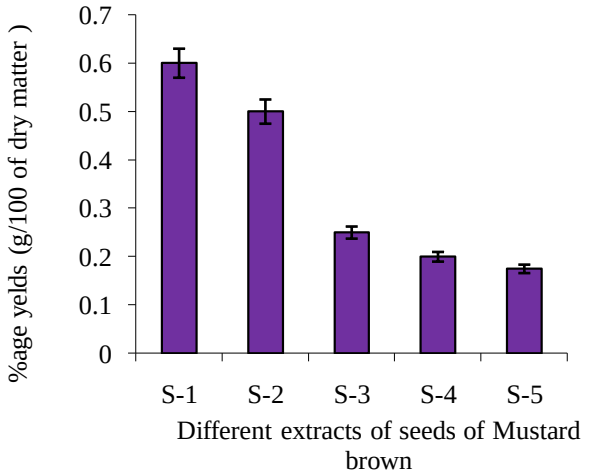

Figure 1: %age yield of extract of Mustard brown seed and its different fractions

3.2 Antibacterial activity of Brown mustard

Antibacterial activity was investigated by applying different fractions and extracts of Mustard brown seeds obtained by *n*-hexane (S-2), chloroform (S-3), ethyl acetate (S-4) and acetone (S-5) and extract of methanol (S-1) in counter to three bacteriological strains i.e. *E. coli, B. subtilis* and *Rhodococcus spp.* Figure 2 presented the graphical representation of the antibacterial activity of different extract and fractions of Mustard brown seed against

Escherichia coli (a), *Bacillus subtillus* (b) and *Rhodococcus* (c). Figure 3 displayed the antimicrobial activity of methanol extract of Brown mustard seed against *Escherichia* coli (a), *Bacillus subtillus* (b) and *Rhodococcus* (c) Antibacterial action exhibited substantial lessening of bacteriological development which was indicating in the well in term of area of inhibition and area of inhibition quantified with vernier calliper. Methanol extracts (S-1) showed maximum activity against *Bacillus subtillus* which was about 29 mm in diameter. Activity of methanol extract against *Escherichia coli* was about 25 mm while it showed 22 mm activity against *Rhodococcus*. *n*-hexane fraction (S-2) of seeds of Mustard brown showed maximum activity against *Escherichia coli* with 20 mm size of diameter and showed 19 mm as well as 18 mm activity against *Bacillus subtillus* and *Rhodococcus* respectively.

Chloroform fraction (S-3) of plant seeds showed maximum activity against *Rhodococcus* which was about 16 mm while it showed 15 mm activity against both *Bacillus subtillus* activity as well as *Escherichia coli*. Ethyl acetate fraction (S-4) of Mustard brown showed maximum activity against *Rhodococcus* which was about 10 mm in diameter while its activity again *Bacillu ssubtillus* and *Escherichia coli* was very low about 9 mm and 8 mm size of inhibition zone respectively. Acetone fraction (S-5) of seeds of Mustard brown showed maximum activities against *Bacillus subtillus* which was 18 mm while against *Escherichia coli* and *Rhodococcus* it exhibited 12 and 14 mm respectively. Kanamysin (S-6) i.e. positive control showed size of inhibition zone of 26 mm against *Escherica coli*, 28 mm against *Bacillus subtillus* and 24 mm against *Rhodococcus*. The antibacterial potential of Mustard brown in counter to both gram positive and gram negative bacteria showed that antibiotic composites present in the plant Mustard brown. Standard drug and methanol extract of Mustard brown founded in close agreement with respect to inhibition of bacterial growth in three types of bacterial strains.

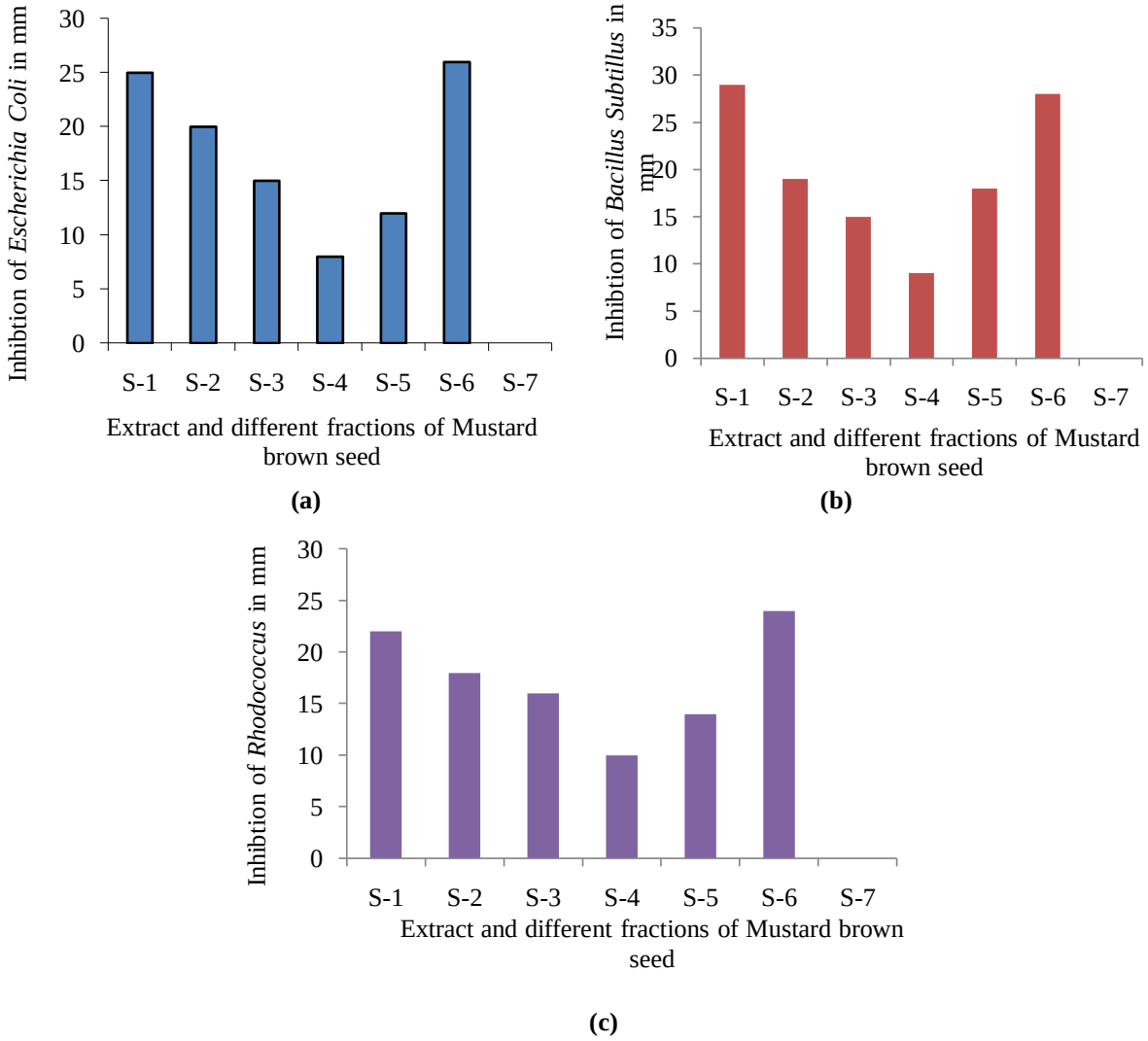

Figure 2: Antibacterial activity of different extracts and fraction of Mustard brown seed against *Escherichia coli* (a), *Bacillus subtillus* (b) and *Rhodococcus* (c)

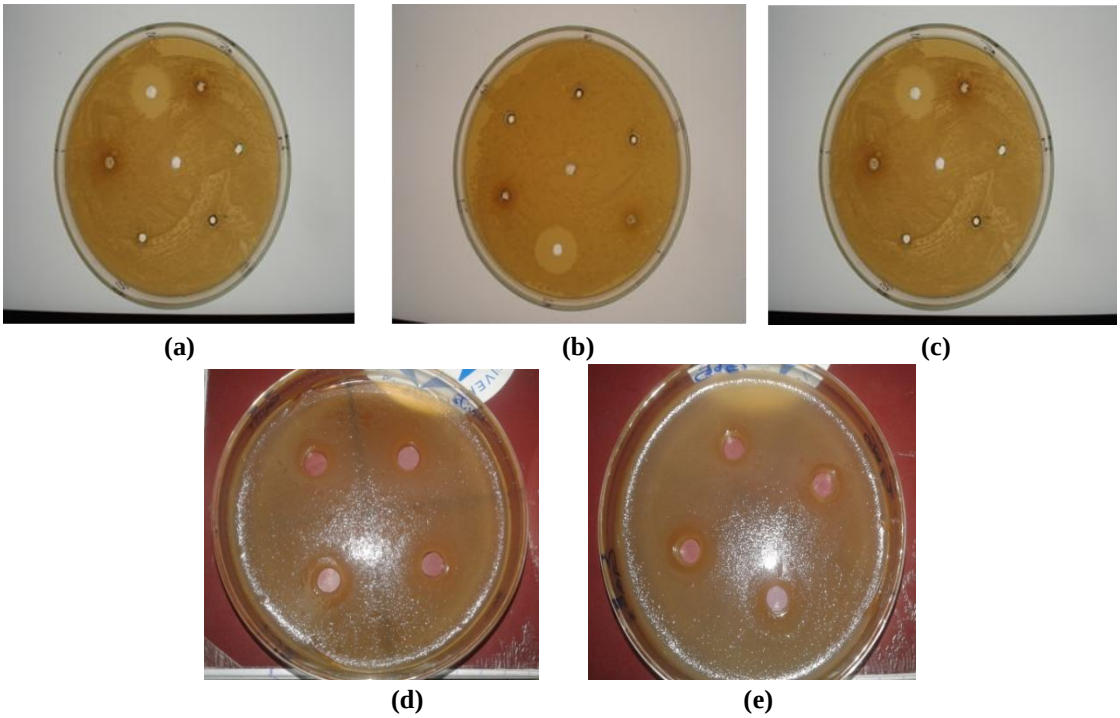

Figure 3: Antimicrobial activity of methanol extract of Brown mustard seed against *Escherichia coli* (a), *Bacillus subtillus* (b) *Rhodococcus* (c), Control (d) and Standard (e)

3.3 Antifungal Activity of Mustard brown:

Fraction of acetone (S-5) showed maximum inhibition zone of 14 mm while extraction of methanol (S-1) and fraction of ethyl acetate (S-4) showed activity of 1 mm and fraction of *n*-hexane (S-2) showed minimum activity which is 0.6 mm while antifungal activity did not express by the fraction of chloroform (S-3) and Terbinafine hydrochloride (S-6) which is standard medicine, presented 19 mm of inhibition of zone. Figure 4 represented the antifungal activity of different fractions and extract of seeds of Mustard brown against *Aspergillus niger*. *Aspergillus niger* is one of the utmost communal species of the genus *Aspergillus*.

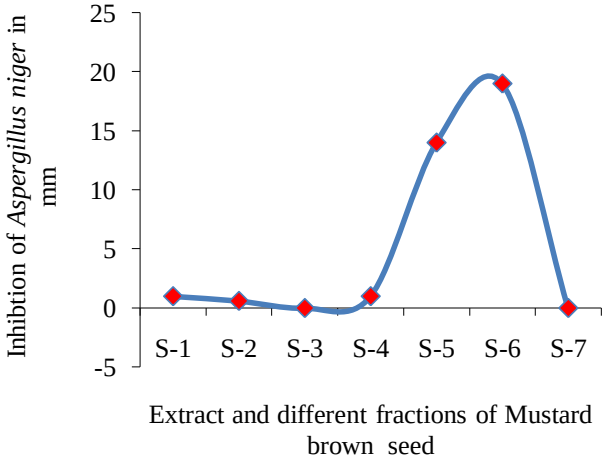

Figure 4: Antifungal activity of extract and fractions of Mustard brown seed against *Aspergillus niger*.

It is pervasive in top soil and is generally pronounced from internal atmospheres, where its dark societies can be disordered with those of *Stachybotrys*. Some *Aspergillus niger* strains have been testified to harvest powerful ochratoxins but other spring differ, appealing this description is based upon false identification of the mycological class. Fraction of *n*-hexane (S-2) has shown greater antifungal activity of 23 mm and other fractions and extract did not express any antifungal activity against *Aspergillus flavus*. Terbinafine hydrochloride (S-6) which is standard drug has exposed 54 mm inhibition area. Figure 5 have been shown the antifungal activity of extract and fractions of Mustard brown against *Aspergillus flavus* and *Trichoderma harzianum. Aspergillus flavus* is a communal fungus in the

atmosphere, and causes storing complications in warehoused. It can also be humanoid pathogens, allied with lungs aspergillosis and occasionally responsible for nasoorbital, otomycotic, and corneal contaminations. Numerous strains harvest momentous numbers of aflatoxin, carcinogenic and extremely poisonous composites.

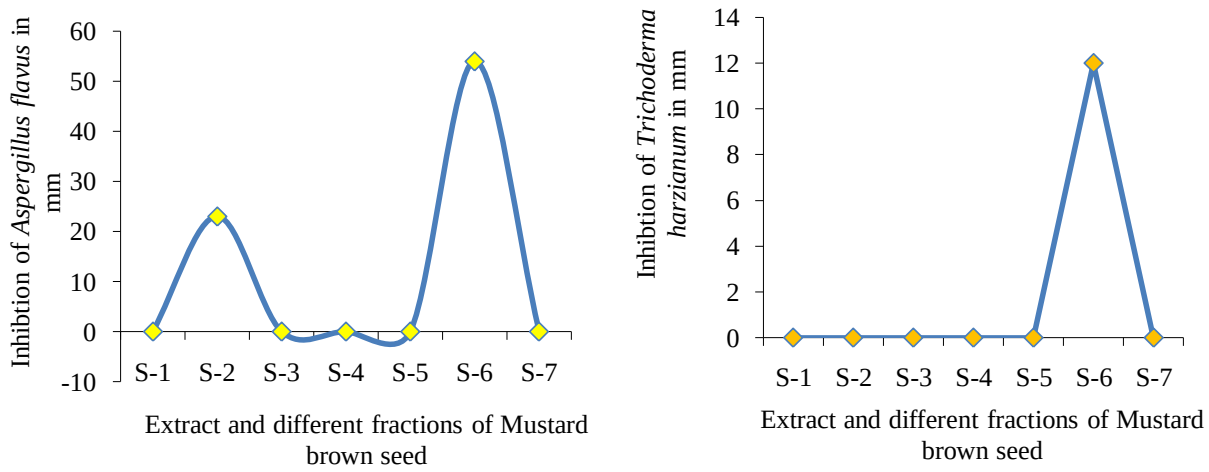

Figure 5: Antifungal activity of extract and fractions of Mustard brown seed against *Aspergillus flavus* and *Trichoderma harzianum*.

The data analysis exposed that fractions and extract of Mustard brown seed did not show any inhibition zone against *Trichoderma harzianum* while standard drug which is Terbinafine hydrochloride (S-6) has shown inhibition zone of 12 mm. *Trichoderma harzianum* is a mushroom that is also cast-off as a fungicide. It is employed for feeding impletetion in plants, curement of seed and treatment of soil by obliteration of numerous ailments producing mycological pathogens. Figure 7 exhibited the antifungal activities of methanol extract of Mustard brown seed against *Aspergillus niger* (a), *Aspergillus flavus* (b) and *Trichoderma harzianum* (c). Guan and Chye [26] revealed that *Brassica juncea* BjCHI1 displays antifungal assets in counter to phyto-pathogens i.e *Ascochytarabiei* & *Colletotri- chumtruncatum* and Present result showed strong antifungal activity against different antifungal strains *Aspergillus niger*, *Aspergillus flavus* and against *Trichoderma harzianum* they did not show activity. Indikar and Desale [27] studied and authenticating a conceivable antibacterial influences of oil gotten from the germs of *Brassica nigra*.

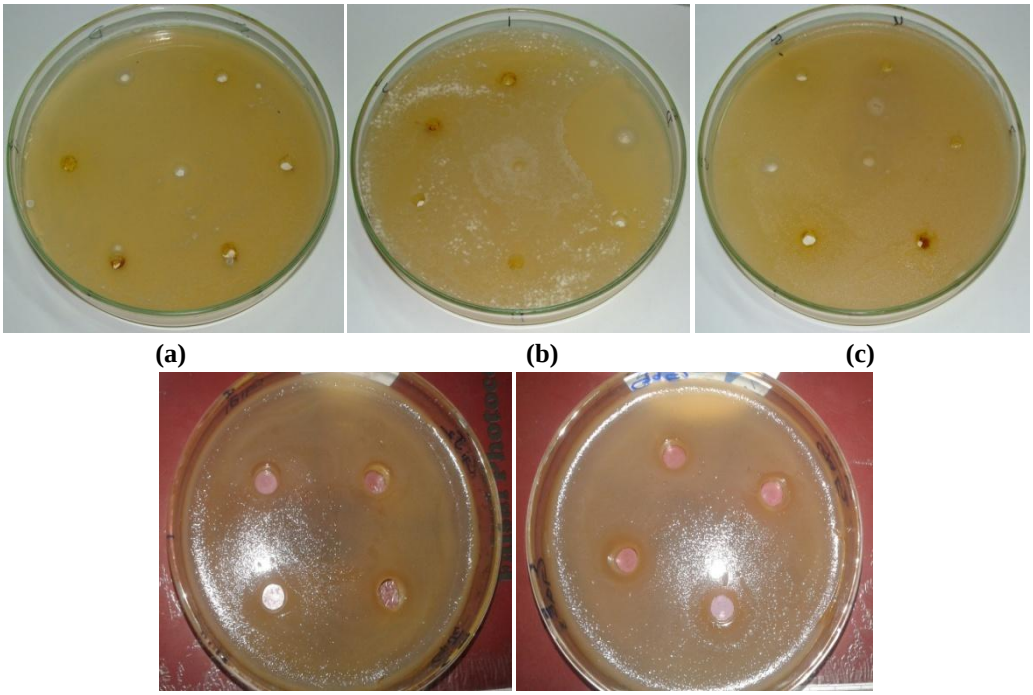

Figure 6: Antifungal activities of methanol extract of Mustard brown seed against *Aspergillus niger* (a), *Aspergillus flavus* (b) *Trichoderma harzianum* (c), Control (d) and Standard (e)

3.4 GC-MS Analysis of Essential Oil

The % yield of essential oil of Mustard brown was founded 0.40%. The chemical substances present in essential oil of plant recognized by GC-MS analysis that were given in Table 1. GC-MS analysis exhibited that seeds of Mustard brown comprehends plentiful compounds. Major components which are determined in essential oil were sinalbins A (4.51%), sinalbins B (2.99%), sitosterol (1.25%), campesterol (1.10%), apigenin (4.51%), chalcone (6.25%), carbohydrates (75%), ash (2.1%), thiocyanate glycosides (1.10%) protein (1.19%). Literature revealed that thiocyanate glycosides, Sinalbin A, Sinalbin B and sinigrin are that components responsible for pungent aroma of seeds of mustard brown. Glucosinolates produced distinct aroma by its hydrolyzation with the myrosinase and as a result produce flavor-active isothiocyanates. Similarly sinalbin produced 4-hydroxybenzyl isothiocyanate, whereas sinigrin vintages the allyl isothiocyanate that are accountable for the bitter aroma [28].

Table 1: Chemical Compounds recognized in the essential oil of seeds of Mustard brown and their percentage area.

Compounds	Retention Index (RI)	Percentage Area (%)
sinalbins A	943	4.51
sinalbins B	950	2.99
Sitosterol	960	1.25
Campesterol	949	1.10
Apigenin	988	4.51
Chalcone	1030	6.25
Carbohydrates	1039	75
Thiocyanate glycosides	1044	1.10
Protein	1055	1.19
Ash	1067	2.1

Mode of Authentication = RI and comparison of mass spectra

4. Conclusion

Mostly extracts of plants comprehend such constituents that demonstrate hindering stroke against diverse microbes like bacteria and fungi. In current research work, it can be concluded that the plant extract and fractions in different solvents which were tested in counter to fungi and bacteria disclosed significant potential as antibacterial as well as antifungal. Methanol extract and *n*-hexane fraction of plant showed highest antibacterial potential against *Bacillus subtilis*, *E. coli* and *Rhodococcusspp* respectively while *n*-hexane fraction showed maximum activity against *Aspergillus niger* and *Trichoderma harzianum* and *n*-hexane fraction showed activity against *Aspergillus flavus*. So it can be indicated from current study, Mustrad brown is a assorted plant with plentiful innovative enactments and would be a healthier ancillary of exceptionally prospective phytotherapeuticals. This learning epitomizes the initial report on the antimicrobial potential of different fractions and extract against both bacterial and fungal strains. Additional studies are acclaimed that will involve various parts of the plant from distinct areas, select different fraction of extracts and purify the most active antimicrobial components. It is determined that herbal drugs are imperative spring for detection of novel mediators for the curement of numerous ailments that are allied to bacteria as well as fungi. The consequences acquired from current research work revealed that extract and fractions of seeds of Mustard brown exhibited the antibacterial activity in comparison to standard Kanamysin in counter to *B. subtilis*, *E. coli* and *S. aureus*. Henceforth Mustard brown extract and fractions can be proving a miracle medication for numerous types of contaminations produce by bacteria. The outcomes in current research study also explored effective antifungal activity by Mustard brown extract and fractions in comparison to standard Griseofulvin.

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