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Efficacy of Phytochemical Constituents of *Castor* Essential oil Towards the Mucor-Mycotic Mold *Cunninghamella Bertholletiae*

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Abstract

The aim of this experiment is to study the efficacy of phytochemical constituents of *Castor* essential oil towards the mucor-mycotic mold *Cunninghamella bertholletiae.* The standard chemical analytical methods were used for the rapid study of the phytochemical constituents responsible for the antimicrobial efficacy of the procured castor essential oil. The standard antimicrobial assay technique employed to study the comparative values of the efficacy of the procured castor essential oil with that of the standard antifungal chemical agents against the clinical isolates obtained from the immune suppressed patients samples of *Cunninghamella bertholletia* mold mucor-mycotic infections. The best susceptibility values recorded in the standard antifungal agents against the clinical isolates of *Cunninghamella bertholletiae* was with Amphotericin B showing the average zone of inhibition diameter of 20.66 mm with the average MIC value of, 1.66 (µ/ml) but the antimicrobial assay results for the *Castor* essential oil showed better values with an average disc diffusion of 22.44mm zone of inhibition diameter with average MIC value of 1.72 µ/ml .This study has shown that the phytochemical compounds present in the *Castor* essential oil proves to be more an effective alternative antifungal substance towards the clinical isolates of *Cunninghamella bertholletiae*.

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Introduction

The mucor - mycosis infections one of the emerging potential dangerous infections among the immune-suppressed patients undertaking treatment for the leukemia, or diabetes with a high mortality rate ranging from 96% for disseminated mucor mycos, 76% for pulmonary infections and 46% for synovial infections[1-3]. The mortality rate depends upon the condition of the patient and the site of infection. The mucor - mycosis was formerly known as zygomycosis which are rare fungal infections targeting the immuno compromised host among the humans [4,5]. In the recent past due to the abuse of antibiotic usage ignoring the constant alarming alert protocols of the WHO, these types of infections are paying way as a potential emerging threat to the human lives. The mucor-mycotic infections were due to the mucormycotic saprophytic fungal molds of the order Mucorales belonging to the family *Mucaraceae* [6-8]. The most common causative agents for these type of mucor –mycotic infections were species from the *Mucor, Absida, Rhizopus* and *Cunninghamella.* The rhinocerebral mucor-mycosis along with the cutaneous mucor- mycosis and the pulmonary mucor-mycosis were frequently found with the inhalation of spores of the *Cunninghamella bertholletia* mold which are heat resistant up to 50^0C posing a potential threats these opportunistic fungal infections are becoming common among the immune suppressed individuals[9,10]. The spores of the mold *Cunninghamella bertholletia* been transported through the blood to the different parts of the human body causing the necrosis of the tissue among the immune suppressed patients. The infection of the eye ulceration or disfiguration of the face were also reported along with the gastrointestinal infections [1-8]. Though, the standard of antifungal-based therapy for *Cunninghamella bertholletia* infections is available for the treatment of this mold, only 33% of the recovery rate recorded. Hence, the need of the hour is to find an alternative potential prophylaxis therapy by revoking the forgotten ancient natural herbal medicine remedy to support the modern antifungal therapy in the treatment of the *Cunninghamella bertholletia* mold infections [5-10]. There are versatile of natural herbal products in the form of essential oils were used in the ancient medicine in the treatment of various dangerous pathogens of bacterial, fungal, and viral infections to a great results [11-13]. This study is focused on the isolation and purification of the clinically procured aseptic samples of the fungal mold *Cunninghamella bertholletia* from the immune suppressed patients and to check its susceptibility with the efficacy of one such essential oils. The essential oil chosen for this study was *Castor* essential oil procured from the local market. The castor essential oil been extracted from the beans of the *Ricinus communis* belonging to the perennial flowering plant of *Euphorbiaceae* spurge family [14-16]. The castor essential oil contains a rich source of phenolic compounds along with the other chemical constituents which possess to be a great antimicrobial agent[14-18] and qualifies for this study. In this study the efficacy of the procured castor oil from the local market was tested against the clinical isolates obtained from the immune suppressed patients samples of *Cunninghamella bertholletia* mold mucor-mycotic infections [1-10]. The standard chemical analytical methods were used for the rapid study of the phytochemical constituents responsible for the antimicrobial efficacy of the procured castor essential oil. The standard antimicrobial assay technique employed to study the comparative values of the efficacy of the procured castor essential oil with that of the standard antifungal chemical agents against the clinical isolates obtained from the immune suppressed patients samples of *Cunninghamella bertholletia* mold mucor-mycotic infections [1-6].

Materials and Methods

Materials

Castor essential oil procured from Jeddah local market, clinical skin scrape samples from the patient, Sabaraud's Dextrose agar, potassium hydroxide, peptone, lacto phenol. Standard antibiotics and standard Hi-Media were used. All the chemicals used during this investigation were of analytical grade.

Isolation and Purification of Cunninghamella Bertholletiae

The clinical sample from the patients was collected by employing the aseptic scraping technique and was inoculated on a sterile Sabaraud's dextrose agar plate and incubated at 45ºC for 24-48 hours to observe the fungal mold rapidly growing white to tannish-gray color loose cottony colonies. The

colonies mature upon further incubation for 96 hours [19-21]. The high temperature incubation at 45ºC, the heat resistant *Cunninghamella bertholletiae* gets isolated from the other species of *Cunninghamella* which are all heat sensitive[7-10]. The potassium hydroxide and lacto phenol test was performed to observe the mold hyphae under microscope by employing the wet mount technique as the confirmatory test. The microscopic observation reveals the sporangiophores, terminal vesicles with nonseptate or sparsely septate broad hyphae and the presence of oval shaped sporangioles with the sporangiospores with tuberculate projections[4-10].

Antimicrobial Susceptibility Test

The antimicrobial susceptibility test for the isolated clinical specimens of *Cunninghamella bertholletiae* were evaluated for the efficacy of the standard synthetic chemical antifungal agents by performing the latest rapid e-test methodology where the clinical isolates were inoculated on Sabaraud's dextrose agar plates separately and e-test plastic strips for the respective antibiotics were impregnated and incubated at 45ºC overnight to visualize the zone and ellipse and the results were tabulated by interpreting the observed results for the interaction of the ellipse as the Minimum inhibitory Concentration (MIC) whereas the zone as the susceptibility of the antibiotic towards the mold [3,4,5,22]. The traditional standard antibiotic assay methods such as Kirby-Bauer disc diffusion method was employed to observe the susceptibility of the clinical isolates of *Cunninghamella bertholletiae* the standard disc prepared from the *Castor* essential oil extract where the mold isolates were inoculated separately on the Sabaraud's dextrose agar plates along with the impregnated discs for 24 hours at 45ºC to observe the zone formation determining the sensitivity of the mold towards the disc[22-25]. The results were tabulated and interpreted. The MIC values along with Minimum Fungicidal Concentration (MFC) values for the efficacy antimicrobial activity of the *Castor* essential oil towards the mold was estimated by performing the standard tube dilution method where the clinical isolates were inoculated separately in the different sets of dilutions of the essential oil in the peptone water and incubated for 24 hours at 45ºC to observe the no turbidity determining the sensitivity of the mold towards the acid [22-25]. The last dilution with turbidity determines the MIC value of the acid towards the mold. The results were tabulated and interpreted. The MFC was determined by inoculating each dilution of MIC dilutions onto the separate agar plates for each clinical isolates of *Cunninghamella bertholletiae* for the MIC dilutions separately. The inoculated plates were incubated for 24 hours at 45ºC to observe the no growth determining the sensitivity of the mold towards the *Castor* essential oil. The first dilution with no growth determines the MFC of the essential oil towards the fungal mold. The results were tabulated interpreted.

Phyto-Chemical Analysis of the Castor Essential Oil

The phyto-chemical properties analysis of the *Castor essential oil* procured from the local market was determined by the following methodologies[14-18,25-27].

Biochemical Analysis for Alkaloids

Mayer's Test

Castor essential oils were mixed with a drop of mercuric chloride and potassium iodide respectively resulting in the formation of a creamy substance indicating the presence of alkaloids.

Wagner's Test

A drop of *Castor* essential oil was mixed with a drop of potassium iodide and iodine resulting in the formation of a reddish brown precipitate which indicates the presence of alkaloids in the oil.

Biochemical Analysis for Reducing Sugar

Fehling's Test

2 ml of Fehling's reagents A and B were mixed with the *Castor* essential oil in a test tube and heated slightly to observe brick red color indicating the presence of reducing sugar.

Benedict's Test

In a test tube 2 ml of Benedict's reagent was treated with the *Castor* essential oil and heated gently heated to observe the formation of orange red precipitate indicates the presence of reducing sugar

Biochemical Analysis for Steroids

Salkowski's Test

The*Castor* essential oils were mixed with 2ml of

chloroform along with 2 ml of concentrated sulphuric acid in a test tube and gently shaken to observe a reddish brown color which indicates the presence of steroids.

Chloroform and Sulphuric Acid with Acetic Acid Mixture Test

The Castor essential oil was treated with a mixture of 2ml of chloroform and concentrated sulphuric acid with 2 ml of acetic acid resulting in the formation of a green colour which indicates the presence of steroids

Biochemical Analysis for Proteins

Ninhydrin Test

The *Castor* essential oils were mixed with 2 ml of ninhydrin solution and heated gently to observe a violet color indicating the presence of protein

Xanthoproteic Test

The *Castor* essential oils were treated with a few drops of concentrated nitric acid resulting in the formation of a yellow colour which indicates the presence of proteins

Biochemical Analysis for Phenol

Litmus Test

A drop of *Castor* essential oil was added to the blue litmus paper which turns red in color due to acidic nature indicating the presence of phenol.

Phthalein Dye Test

The *Castor* essential oils were treated with conc. sulfuric acid after heating with phthalic anhydride results in the formation of colorless condensation compound and the addition of dilute sodium hydroxide solution results in the formation of a pink color fluorescent compound which indicates the presence of phenol.

Ferric Chloride Test

Castor essential oils were boiled with 10 ml of water in a test tubes. A few drops of ferric chloride was added to the 10 ml of heated *Castor* essential oil in a test tube to observe a blue black coloration which indicates the presence of phenol

Biochemical Analysis for Glycosides

Libermann-Burchard's Test

The mixture of 2 ml of acetic acid with 2 ml of chloroform was treated with the *Castrol* essential oil in a test tube and few drops of concentrated sulphuric acid was added by placing the test tube on ice to observe the color change from violet to bluish green which indicate the presence of glycosides

Keller-kilani Test

Castrol essential oil was treated with 2ml of glacial acetic acid with 1 to 2 drops of ferric chloride solution in a test tube and 2 ml of conc. sulphuric acid was added to observe a brown ring at the interface which indicates the presence of cardiac glycosides.

Biochemical Analysis for Amino Acids

Ammonia Test

Dilute ammonia and conc. sulphuric acid treated with aqueous *Castrol* essential oil in a test tube to observe yellowish color formation indicating presence of amino acids.

Biochemical Analysis for Flavonoids

Ammonia and H_2SO_4 Mixture Test

The *Castor* essential oil was treated with dilute ammonia and conc. sulphuric acid resulting in the formation of a yellow colour which indicates the presence of flavonoids.

Biochemical Analysis for Iodine

Iodine Test

The *Castor* essential oil was determined added with a 2ml of iodine solution which results in the positive purple colored test which indicates the presence of iodine.

Biochemical Analysis for Terpenoids

Chloroform and H_2SO_4 Mixture Test

The Castor essential oil was treated with 2ml of chloroform and concentrated sulphuric acid resulting in the formation of a brownish red layer which indicates the presence of terpenoids

Results and Discussion

A Comparative analysis study was performed for the antimicrobial efficacy of *Castor* essential oil extract with that of the standard antifungal agents towards the clinical isolates of *Cunninghamella bertholletiae* [3-8]. The antimicrobial assay results for the *Castor* essential

oil procured from the local market shown significant antimicrobial activity results against all the clinical isolates of *Cunninghamella bertholletiae* with an average disc diffusion of 22.44.mm zone of inhibition diameter determining the susceptibility obtained from performing the Kirby-Bauer technique with an average MIC value of 1.72 µ/ml and an average MFC value of 2..30 µ/ml. The best susceptibility for the clinical isolates of *Cunninghamella bertholletiae* towards the *Castor* essential oil was observed from the throat swab isolates of *Cunninghamella bertholletiae* sample with a zone diffusion of 28 mm with MIC of 2.25 µ/ml and MFC of 2 .5 µ/ml whereas the least susceptibility was observed from the nail scrape isolates of *Cunninghamella bertholletiae* sample with a zone diffusion of 18 mm with MIC of 1.75 µ/ml and MFC of 2 µ/ml respectively [12,13,17]. The susceptibility with MIC and MFC results of the other isolates of *Cunninghamella bertholletiae* sample towards the *Castor* essential oil were also shown satisfactory results when compared with that of the standard antifungal agents against clinical isolates of *Cunninghamella bertholletiae*. The results of the other isolates of *Cunninghamella bertholletiae* towards the *Castor* essential oil obtained were ranged for the susceptibility with a zone diameter from 18 to 28 mm in disc diffusion method with MIC from 1 .5 to 2.5 µ/ml and MFC of 1.75 to 2.75 µ/ml respectively. The efficacy of the *Castor* essential oil extract against clinical isolates of *Cunninghamella bertholletiae* has shown excellent results when compared with that of the standard antifungal agents used in therapy. The average value of the zone of inhibition susceptibility value of the *Castor* essential oil extract against clinical isolates of *Cunninghamella bertholletiae* was 22.44 mm for all the clinical isolates compared to the standard antifungal agents values of 14.88 mm for Voriconozole, 10.11 mm for Itraconazole , 20.66 mm for Amphotericin B, 11.11 mm for Fluconazole, , 16.22 mm for Posoconazole, 16.11 mm for Metronidazole, 16.55 mm for Ketoconazole and 10.44 mm for Rifampin respectively for all the samples assayed. The average MIC value of the *Castor* essential oil extract against clinical isolates of *Cunninghamella bertholletiae* was 1.72 µ/ml for all the samples compared to the standard antifungal agents values of 1.30 (µ/ml) for Voriconozole, 1.47 (µ/ml) for Itraconazole , 1.66 (µ/ml) for Amphotericin B, 1.75 (µ/ml) for Fluconazole, 1.94(µ/ml) for Posoconazole, 2.11 (µ/ml) for Metronidazole, 2.30 (µ/ml)for Ketoconazole and 2.41 (µ/ml) for Rifampin respectively for all the clinicals isolates of *Cunninghamell abertholletiae* samples assayed. The best susceptibility values recorded in the standard antifungal agents against the clinical isolates of *Cunninghamella bertholletiae* was with Amphotericin B showing the average zone of inhibition diameter of 20.66 mm with the average MIC value of , 1.66 (µ/ml) but the antimicrobial assay results for the *Castor* essential oil showed better values with an average disc diffusion of 22.44mm zone of inhibition diameter with average MIC value of 1.72 µ/ml. The details of the obtained results were tabulated (Table 1 to 3) for the references. A detailed comparative analysis chart (Figure.1) was prepared for the antimicrobial activities of *Castor* essential oil extract with that of standard antifungal agents against clinical isolates of *Cunninghamella bertholletiae* for the references. The phytochemical analysis study was also been conducted for the procured *Castor* essential oil to determine the constituents which are responsible for the antimicrobial efficacy. The phytochemical analytical tests conducted for the *Castor* essential oil were as shown in Table 4. The obtained interpretation from the phytochemical analytical test results showed the presence of chemical compounds such as alkaloids, flavonoids, steroids, proteins, phenols, glycosides, reducing sugar, iodine, amino acids and terpenoids respectively. The phytochemical test results were tabulated for the reference (Table 4). The presence of the vital phytochemical component in the *Castor* essential oil is the phenolic compounds which serves as a potential antimicrobial activity and shown the promising results against the clinical isolates of *Cunninghamella bertholletiae* when demonstrated with the standard antimicrobial assay techniques[14-17]. The presence of pheonolic and other miscellaneous constituents in the *Castor* essential oil extract contributes to its rich antimicrobial content and has shown the promising results in this study as well. Tab 2-3

Conclusion

The phytochemical compounds present in the *Castor* essential oil acts as an effective remedy towards

Table 1. Comparative chart of antimicrobial sensitive activities standard antifungal agents against clinical isolates of *Cunninghamella bertholletiae by* e-test study

Standard Anti-fungal agents	Specimens									Average Zone value
	Sinovial fluid	Nasal swab	Pleural effusion	Nail scrape	Abscess swab	Oral cavity	Throat swab	Ulcer swab	Wound swab	
Voriconazole	20 mm S	20 mm S	9 mm I	11 mm I	25 mm S	22 mm S	4 mm R	3 mm R	20 mm R	14.88 mm
Itraconazole	12 mm I	3 mm R	4 mm R	3 mm R	20 mm R	12 mm I	13 mm I	4 mm R	20 mm S	10.11 mm
Amphotericin B	25 mm S	14 mm I	23 mm S	26 mm S	20 mm S	22 mm S	20 mm S	24 mm S	12 mm I	20.66 mm
Fluconazole	10 mm I	10 mm I	21 mm S	11 mm I	9 mm I	13 mm I	12 mm I	12 mm I	2 mm R	11.11 mm
Posaconazole	20 mm S	20 mm S	9 mm I	11 mm I	21 mm S	11 mm I	23 mm S	21 mm S	11 mm I	16.22 mm
Metronidazole	11 mm I	21 mm S	10 mm I	21 mm S	20 mm S	20 mm S	9 mm I	11 mm I	22 mm S	16.11mm
Ketoconazole	14 mm I	2 mm R	11 mm I	21 mm S	20 mm S	21 mm S	20 mm S	10 mm I	20 mm S	16.55 mm
Rifampin	6 mm R	4 mm R	12 mm I	23 mm S	6 mm R	4 mm R	4 mm R	14 mm I	12 mm I	10.44 mm
Total Sensitives	3	3	2	4	5	4	3	2	3	
Total Intermediates	4	2	5	3	1	3	3	4	3	
Total Resistance	1	3	1	1	2	1	2	2	2	

Table 2. Comparative MIC values chart of antimicrobial activites of standard antifungal agents against clinical isolates of *Cunninghamella bertholletiae* by e-test study

Standard Antifungal agents	Specimens									Aver-age MIC Values
	Sinovi-al Fluid	Nasal Swab	Pleural Effusion	Nail Scrape	Ab-scess swab	Oral cavity	Throat swab	Ulcer swab	Wound swab	
Voriconazole	1.5 µ/ml	1.25µ/ml	1.75 µ/ml	1.25 µ/ml	1.75 (µ/ml)	1.5 µ/ml	1.25 µ/ml	1.5(µ/ml)	1.25µ/ml	1.30 (µ/ml)
Itraconazole	1.5 µ/ml	1.25 µ/ml	1.75 µ/ml	1.25 µ/ml	1.75 (µ/ml)	1.5 µ/ml	1.5 µ/ml	1.5 (µ/ml)	1.25 µ/ml	1.47 (µ/ml)
Amphoteri-cin B	1.75 µ/ml	1.5 µ/ml	1.75 µ/ml	1.5 µ/ml	2 µ/ml	1.75 µ/ml	1.5 µ/ml	1.75 (µ/ml)	1.5 µ/ml	1.66 (µ/ml)
Fluconazole	1.75 µ/ml	1.5 µ/ml	2 µ/ml	1.5 µ/ml	2.25 µ/ml	1.75 µ/ml	1.75 µ/ml	1.75 (µ/ml)	1.5 µ/ml	1.75 (µ/ml)
Posacona-zole	1.75 µ/ml	1.75 µ/ml	2.25 µ/ml	1.75 µ/ml	2.25 µ/ml	2 µ/ml	2 µ/ml	2 (µ/ml)	1.75 µ/ml	1.94 (µ/ml)
Metronida-zole	2 µ/ml	2. µ/ml	2.25 µ/ml	1.75 µ/ml	2.5 µ/ml	2µ/ml	2.25 µ/ml	2.25 (µ/ml)	2. µ/ml	2.11 (µ/ml)
Ketocona-zole	2.25 µ/ml	2.25 µ/ml	2.5 µ/ml	2 µ/ml	2.75 µ/ml	2.µ/ml	2.25 µ/ml	2.5 (µ/ml)	2.25 µ/ml	2.30 (µ/ml)
Rifampin	2.25 µ/ml	2.25 µ/ml	2.5 µ/ml	2 µ/ml	2.75 µ/ml	2.25 µ/ml	2.5 µ/ml	2.75 (µ/ml)	2.5 µ/ml	2.41 (µ/ml)

Table 3. Efficacy of *Castor* essential oil extract against Clinical isolates of *Cunninghamella bertholletiae*

	Castor essential oil extract		
Specimen	Disc Diffusion	MIC	MFC
Sinovial fluid	19 mm S	1.5 µ/ml	1.75 µ/ml
Nasal swab	25 mm S	1.5 µ/ml	1.75 µ/ml
Pleural effusion	23 mm S	1.75 µ/ml	2 µ/ml
Nail scrape	18 mm S	1.75 µ/ml	2 µ/ml
Abscess swab	21 mm S	2 µ/ml	2.25µ/ml
Oral cavity	25 mm S	2 µ/ml	2.25 µ/ml
Throat swab	28 mm S	2.25µ/ml	2.5 µ/ml
Ulcer swab	19 mm S	2..25µ/ml	2.5 µ/ml
Wound swab	24 mm S	2.5 µ/ml	2.75 µ/ml
Average value	22.44mm S	1.72 µ/ml	2.30 µ/ml

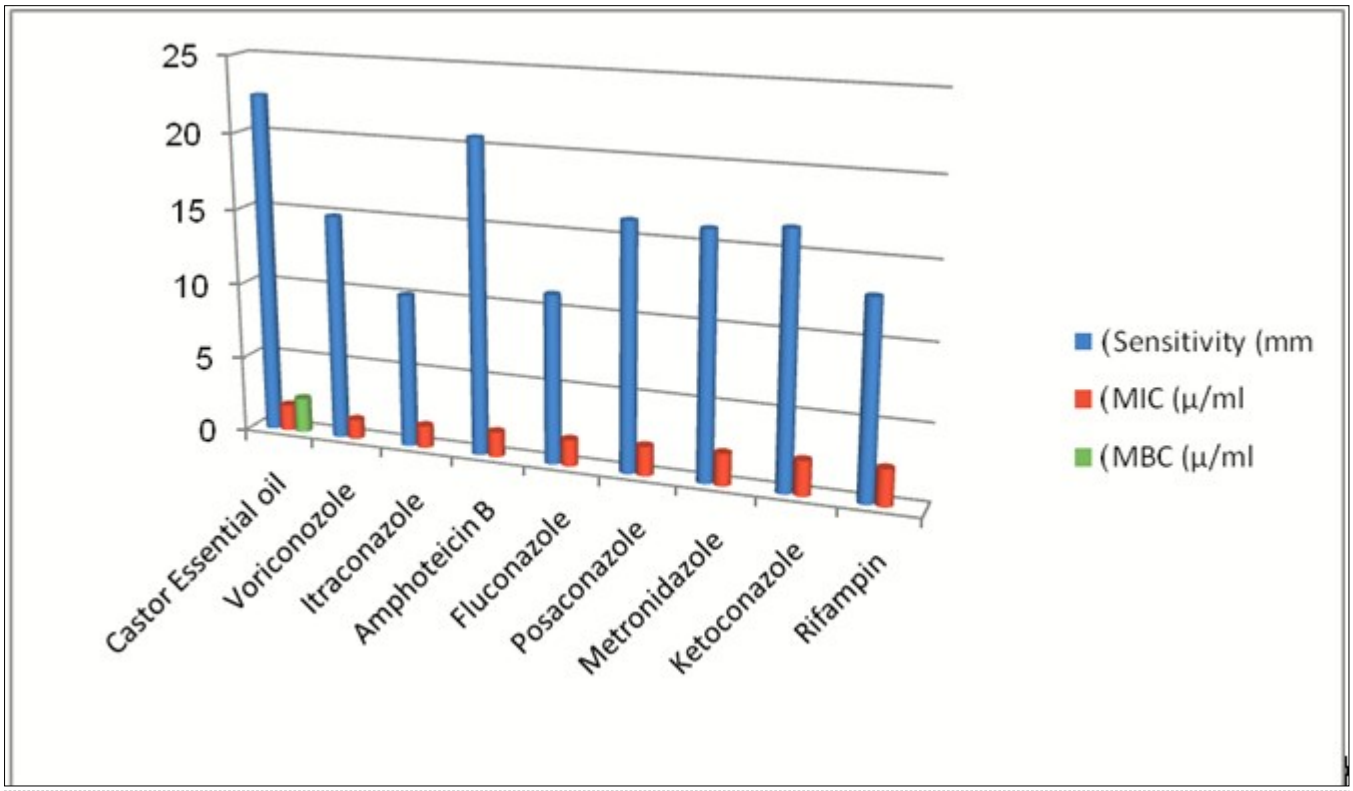

Figure 1. Comparative analysis for the antimicrobial activities of *Castor* essential oil extract versus standard antifungal agents against clinical isolates of *Cunninghamella bertholletiae*

Table 4. Phyto-chemical analysis of the *Castor* essential oil

Biochemical Analysis	Observed Result	Phytochemical constituents present
Mayer'sTest	Creamy Substance	Alkaloids.
Wagner's test	Reddish brown precipitate	Alkaloids.
Fehling'sTest	Brick red color	Reducing sugar
Benedict's Test	Orange red precipitate	Reducing sugar
Salkowski'sTest	Brown color	Steroids
Chloroform and Sulphuric acid with Acetic Acid mixture Test	Green color	Steroids
Ninhydrin	Violet color	Proteins
Xanthoproteic Test	Yellow colour	Proteins
Litmus Test:	Red color	Phenol
Phthalein Dye Test	Pink fluorescent com-pound	Phenol
Ferric chloride test	Blackish blue color	Phenol
Libermann-Burchard'sTest	Bluish green color	Cardiac glycosides
Keller-kilani test	Brown colored ring	Glycosides
Ammonia test	yellow colour	Amino acids
Ammonia and Sulphuric acid mixture Test	yellow colour	Flavonoids
Iodine Test	Purple color	Iodine
Chloroform and Sulphuric acid mixture Test	brownish red layer	Terpenoids

the the clinical isolates of *Cunninghamella bertholletiae* compared to the standard antifungal agents. The interpretation of the observation and results for the *Castor* essential oil showed the promising study results regarding its efficacy as a potential antifungal agents when compared to that of the standard synthetic chemical agents used against the clinical isolates of *Cunninghamella bertholletiae*. This study recommends for more such of natural essential oils from the plant source as an alternative towards the synthetic chemical antimicrobial substances with more detailed studies need to be done in near future with the expectations that many dangerous infections can be cured with these types of phytochemical compounds. Thus, this study has shown that the phytochemical compounds present in the *Castor* essential oil proves to be more an effective alternative antifungal substance towards the clinical isolates of *Cunninghamella bertholletiae*.

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Conflict of Interest

No conflict of interest.

Contribution of Authors

All authors have made substantial contribution to the work and approved it for publication.

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