



Original Research Article

Isolation and Identification of Bacteria from Dried Fermented Leaves of *Cassia obtusifolia* .L (Kawal)

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Abstract

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The present study of *Cassia obtusifolia* leaves (Kawal) were obtained from Darfur region western Sudan (Kutum, Jineena and Zalingie). The objective of this study is to isolate bacteria from different samples of dried fermented *Cassia obtusifolia* leaves (kawal) and to identify bacteria by using different biochemical tests, which is present in natural environment and fermented traditionally as survival, the study was carried out in Khartoum State-Sudan, during February 2019 to September 2019, the samples were collected from three area (Kutum, Jineena and Zalingie). The traditional method for fermentation was used and the bacteria were isolated by using nutrient agar. The biochemical tests were used to determine type of bacteria and the results were cited positive with catalase, citrate, urease, motility tests, while gas and indol were showed negative result, the microorganisms were examined. The sugar fermentation tests for bacteria were recorded positive with glucose, sucrose, mannitol tests, while it showed negative at lactose sugar. The results revealed that all the samples were positive in nutrient agar media and the isolation of microorganisms were showed that, the *Bacillus subtilis* bacteria is only microorganism that was determined in the fermented kawal leave.

Keywords: *Cassia obtusifolia*, Darfur region, Fermentation, Isolation, Kawal.

INTRODUCTION

Cassia obtusifolia is a member of (Leguminosae Family) it is a wild spread African plant, found in waste lands in the rainy season, the leaves of the species can be fermented to (kawal) and is used by people from the eastern part of Chad and the western part of Sudan as meat replacer or meat extender (Dirar, 1993). One method of food transformation and preservation is fermentation process that improves nutritive value, safety, hygienic, organoleptic quality of the food and detoxification of anti-nutrient factors (AFNOR, 1986; Nnam and Obiakor, 2003; Nout et al., 1987; Sawadogo-Lingani et al., 2008; Law et al., 2011). In Africa, the art of fermentation is widespread including the processing of fermented cereals, legumes, the fermented cereals

are dominated by Lactic Acid Bacteria (LAB) associated with yeast, *Bacillus* species one of the microorganisms that dominates, with their major proteolytic activity to hydrolyze proteins into amino acids and ammonia (Ayo, 2010; Kuboye, 1985). It play an important role in providing food security, improving the nutrition and social well-being of millions of people around the world (Odufa, 1985). They also play a very important role in the socioeconomics of developing countries. The process generally produced from plant or animal-based raw materials in combination with fungi or bacteria, which are either present in the natural environment, or added intentionally by human to obtain the desirable end-products (Law et al., 2011).

Objective

The main objective of the present study is to isolate bacteria from different samples of dried fermented of *Cassia obtusifolia* leaves (kawal) and to identify bacteria by using different biochemical tests.

MATERIALS AND METHODS

Study area and collection of sample

This study was carried out in Darfur region, western Sudan, it covers an area of 493,180 square kilometers (190,420 sq mi). It is largely an arid plate with the Marrah Mountains (Jebel Marra), a range of volcanic peaks rising up to 3,042 meters (9,980 ft), with rainfall down by up to 30% over 40 years and the Sahara advancing by well over a mile every year, the soil is composed of clay plains in some areas and low hills of sandy known as alkoz and sand stone hills in eastern areas. Dried fermented samples (kawal) were collected from three different places in Darfur region (Kutum, Jineina, Zalingie) from February 2019 to April 2019. All dried powdered samples were transferred to laboratory for microbial analysis.

Preparation of medium

28g was suspended in 100ml of distilled water in conical flask after mixing the solution, it was heated to boiling gently to dissolve the medium completely and sterilized by autoclaving at 121°C for 15 minutes. The medium was allowed to cool down to 45-50 °C, and then poured in sterile Petri dishes.

Isolation of bacteria

5g of powdered kawal samples were dissolved in 95ml sterilized distilled water, 1ml of sample was serially diluted up to 10⁻⁵ dilution. 0.1ml from each dilution was cultured by using streaking method in the nutrient agar medium, the plates incubated at 37 °C for 24 hours. Discrete colonies exhibited smooth, white-creamy color on nutrient agar was sub cultured on new nutrient agar plates to obtain pure cultures. The pure colonies were cultured on nutrient agar in test tubes for further identification by biochemical tests (El-Naggar, 2004).

Identification of bacteria

All suspected isolates of bacteria were subjected to standard biochemical tests for bacterial identification by following the criteria described in {Bergey's Manual of

systematic Bacteriology (Holt et al., 1994) biochemical tests include: (Indole test, urease test, motility test, citrate test, H₂S gas production test, catalase test. Followed by fermentation of (Glucose, Sucrose, Lactose, Mannitol) in addition to gram staining (Holt et al., 1994).

Biochemical tests

Gram staining

Drop of sterile normal saline was placed in sterilized dried microscopic slide under aseptic condition. Pure colony of isolated bacteria of overnight culture was emulsified on drop of normal saline and rolled by sterile loop until thin layer of smear was prepared and allowed to dry at room temperature and fixed by passing the smeared slide rapidly over the flame. After cooling it was covered with crystal violet solution and allowed for one minute and discarded and was washed off with water, then the smear was covered with fresh iodine solution and left for one minute and washed with water, then it was rinsed with absolute alcohol for (15-20 seconds) until remove color appears. And washed with water and covered with Safranin solution for one minute, then rinsed with distilled water and air dried at room temperature and examined microscopically under oil immersion lens, a light microscope at X100. (the isolates were gram positive rods). The gram staining technique was used to categorize the isolates into gram negative and gram positive (Cappuccino and Sherman, 2010).

Indole test

Peptone water medium was prepared according to the manufacturing instructions (Micro master limited, INDIA) and distributed in test tubes, autoclaved at 121 °C for 15 minutes and allowed to cool. Full of suspected bacterial colonies were cultured into the medium and incubated at 37 °C for 24 hours. Five to six drops of Kovac's reagent were added carefully to the culture through the internal surface of the tube. The change in the color of Kovac's reagent from brown to cherry red was considered positive indole production by the organism. (the isolated bacteria were indole negative) (MacFaddin and Jean, 2013).

Urease test

The basal medium was prepared according to the manufacturing instructions (Scharlau) without glucose and urea, sterilized by autoclaving at 121 °C for 15 minutes, cooled to about 50 °C, then sterilized solution

of 20%, 10% urea and glucose respectively were added. The medium was distributed in sterile test tubes and allowed to cool and solidify at room temperature in slant form. Suspected bacterial colonies were cultured in sterile loop into the medium by streaking the slant and incubated at 37 °C for 24 hours. Positive cultures produce an alkaline reaction in the medium showing of change in color. No change in color of the medium indicates urease negative. (the isolated bacteria were urease positive) (Collins et al., 1995).

Motility test

Motility test medium was prepared according to the manufacturing instructions (Hi media laboratories limited, INDIA) and distributed in test tubes, sterilized by autoclaving and allowed to cool then inoculated.

With suspected bacterial by stabbing through the center of the medium with sterile straight needle to approximately one-half the depth of the medium, incubated at 37 °C for 24 hours. Motile organism migrates throughout the medium, which becomes turbid. Non-motile confined to the stab inoculum. The isolated bacteria were motile organisms (Wilson and Miles, 1975).

Citrate utilization test

Simmon's citrate agar medium was used for citrate test. The medium was prepared according to the manufacturing instructions (Scharlau) and distributed in test tubes, sterilized by an autoclave at 121 °C for 15 minutes, then all tubes were slanted, and then allowed to cool and solidify at room temperature. Under aseptic condition pure suspected bacterial colonies were streaked on slanted tubes by sterile loop and incubated at 37 °C for 24 hours. The utilization of the citrate was showed by blue color; whereas non-utilization did not change (the isolated bacteria were citrate positive) (Cappuccino and Sherman, 2010).

Hydrogen Sulphide gas production test

Kliger iron agar (KIA) medium was used for H_2S gas production test, the media was prepared according to the manufacturing instructions (Hi media laboratories limited, INDIA) and distributed in test tubes, sterilized by an autoclave at 121 °C for 15 minutes, then all tubes were slanted, and allowed to cool and solidify at room temperature. Under aseptic condition, tubes of KIA slants were inoculated with suspected bacterial colonies by stabbing the butt and streaking the slant with sterile straight needle and incubated at 37 °C for 24 hours. The tubes were examined for the production of H_2S

(blackening the medium by the organism). (The isolates were gas negative) (Collee et al., 1996).

Catalase test

Under aseptic condition, drops of H_2O_2 solution were placed in dried slide; pure colony of overnight culture of isolated bacteria was emulsified on the drops of H_2O_2 solution and rolled by sterile loop, the enzyme showed by immediate production of gas bubbles. The absence of Catalase enzyme showed by no bubbles (the isolates were catalase positive) (Cappuccino and Sherman, 2010).

Fermentation of sugars

Sugars were prepared according to the methods described by {Bergey's Manual of systematic Bacteriology [10] with some modifications. 0.25g from each sugar was taken and added to 0.5g pancreatic digest, 0.25g NaCl, 0.0009g phenol red and dissolved in 50ml distilled water. Distributed in test tubes containing Durham tubes and closed well with sterilized cotton and foil, and then sterilized by an autoclave at 121 °C for 15 minutes and allowed to cool. Under aseptic condition, tubes were inoculated with suspected bacterial colonies and incubated at 37 °C for three days. (The isolates were lactose negative and glucose, sucrose, mannitol positive) (Holt et al., 1994).

RESULTS

Isolation of bacteria by nutrient agar

All the samples collected from different places were found to be positive growth in nutrient agar.

Identification of bacteria

In gram staining techniques the isolates were found gram positive rod shapes of *bacilli*, some of them have rod shapes in pairs with rounded ends and some have rod shapes in chains with square ends. In biochemical tests the isolates were found positive Catalase, Citrate, Urease and Motility, while Gas and Indole were found negative. In sugar fermentation tests the isolates were negative at Lactose, while were Positive at Glucose, Sucrose and Mannitol. (Tables 1 and 2)

DISCUSSION

Isolation and Identification of bacteria

This study shown prevalence of bacteria in dried fermented kawal by using nutrient agar medium, the

Table 1. Shows the identification of bacteria by using different standard biochemical tests.

Test /medium	Results
Catalase	+
Citrate	+
Urease	+
Motility	+
Gas	-
Indol	-

Table 2. Shows the fermentation of bacteria by using different sugars.

Sugar	Results
Lactose	-
Glucose	+
Sucrose	+
Mannitol	+

results revealed all samples were positive growth in nutrient agar medium, these results are similar to findings reported by previous study but they have used De Man Rogosa and Sharp (MRS) agar medium. The positive rod shapes were bacillus and identified by positive tests of catalase, citrate, urease, motility and positive fermentation of glucose, sucrose, mannitol this is similar to previous study and the results which were correspond to the study were conducted by previous study was showing the bacillus species as dominant bacteria in kawal samples. The only species of bacillus was confirmed to be *B.subtilis* that it is the catalase positive and lactose fermentation negative. The similar findings were also The results show that the *B.subtilis* bacterium shows prevalence in different dried fermented kawal that found in different regions of Darfur. The same findings were also reported by (Dirar, 1993). The isolation of bacterium bacillus subtilis from dried fermented kawal by nutrient agar was found positive. Further work is requiring using molecular tool with enough samples to confirm identification of *B. subtilis*.

CONCLUSION

Fermented Kawal contain considered amount of Bacteria which May help the digestive system.

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