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Antioxidant, Antidiabetic and Antibacterial Activities of Curd Derived from Selected Plants Fortified with *Ocimum tenuiflorum*

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ABSTRACT

Mangifera indica cv. Apple (mango), *Ananas comosus* cv. Sarawak (pineapple) and *Morinda citrifolia* (noni) are associated with the milk-clotting ability. While *Ocimum tenuiflorum* (holy basil) known to have phytochemicals with important biological activities. In this study, the aim was to determine the extent of *O. tenuiflorum* in providing biological activities to the curd achieved by the three milk-clotting plants. A freeze-dried mixture of plant extracts in the ratio of 1:1:1 was prepared from the kernel of *M. indica* and fruits of *A. comosus* and *M. citrifolia* to form a natural milk-clotting agent. The curd was fortified with *O. tenuiflorum*, which was then examined for antioxidant activities utilising the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity and Ferric Reducing Antioxidant Power (FRAP) assay. The anti-diabetic action was determined using an *α*-amylase inhibiting test, whereas the antibacterial activity was determined using the agar well diffusion method on selected bacteria. The results for DPPH, FRAP and alpha-amylase inhibitory assays for the fortified curd with *O. tenuiflorum* showed IC_{50} values of 1.47±3.82 mg/mL, 370.8±0.3 mg GAE/g and 3.19±1.59 mg/mL, respectively. Antibacterial activity was found in the *O. tenuiflorum* fortified curd against two Gram-positive bacteria (*S. aureus* and *B. cereus*) and three Gram negative bacteria (*S. marcescens*, *E. coli* and *A. baumannii*), all with MIC of 2.3 mg/mL. In conclusion, the *O. tenuiflorum* evaluated to enhance the anti-oxidative, anti-diabetic and antimicrobial properties of the curd achieved by the combined effects of *M. indica*, *A. comosus* and *M. citrifolia*.

***Keywords*:** Milk Coagulation, Curd, Basil, Diabetic, Antioxidant, Bacteria.

Introduction

Milk has been converted to dairy products for ages for their health benefits due to the biologically active components they contain, including bioactive peptides, organic acids, vitamins, oligosaccharides, antioxidants, and probiotic bacteria. Curd is a type of dairy product formed from the curdling of milk through the coagulation process. A small amount of rennet or clotting enzyme usually in crude form is added to the milk to coagulate it and create coagulum.[1] Rennet is a calf extract that contains calf chymosin, a clotting agent. Plant extracts have been employed in the production of dairy products.[2] Among many coagulants used in cheese making, plants may play a major role. The reliance on calf rennet for cheese making is becoming less due to many issues including the increasing demand for cheese worldwide.[3] Plants' commercial availability and use in traditional remedies play a significant role as an animal rennet substitute. Nowadays, humans are exposed to pro-oxidants that damage DNA, proteins, carbohydrates, and lipids.[4] Antioxidants are utilized to slow down the oxidation process, which can lead to cancer due to the release of free radicals.[5]
The free radicals in the surroundings eventually make their way into the body and modify structural and functional biomolecules such as protein and DNA. These may cause ailments such as cancer or diabetes if left untreated.[6]

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Diabetes is a chronic illness characterized by a deficit in some hereditary traits and a spike in oxidative stress influencing the pancreas' synthesis of insulin or by the inefficiency of the insulin produced.[7] Increased glucose levels in the blood arise from such deficits, causing harm to many of the body's systems, including the blood vessels and neurons. Diabetes is consuming an ever-increasing share of national and international healthcare costs, with the number of individuals with the illness expected to exceed 700 million by 2045.[8]
Traditional practices for healing account for about 60 – 80% worldwide and a huge population of them rely on medicinal plants.[9] Many plants have been attributed to be useful for diabetic prevention and treatments.[10] Among them included *Ocimum tenuiflorum* having not only the ability to normalize blood glucose levels but also various other potentials including antioxidant, antimicrobial, anxiolytic and antidepressant activities.[11]However only a few plants are recognized with the potential of being an alternative rennet to coagulate milk.[12] In combining the two features, a likely cocktail containing plant coagulants and phytochemically rich herb capable to fortify the curd with medicinal benefits can be achieved.
The current study aims to use a combination of three known plant coagulants: *Mangifera indica*, *Ananas comosus* and *Morinda citrifolia*, to induce milk coagulation enriched with *O. tenuiflorum* to enhance its biological functionalities.

Materials and Methods

Preparation of plants materials for Milk coagulation
Mangifera indica cv. Apple, *Ananas comosus* cv. Sarawak and *Morinda citrifolia* were acquired from markets in Kuantan, Pahang state, Malaysia. *M. indica* kernels were chopped into small pieces and dried in a 50^{o}C oven for 48 hours until completely dry. After that, an electrical grinder was used to crush the dried kernel pieces into a fine powder.

Water was added to a 5% (w/v) kernel powder and gently mixed for 10 hours. The unwanted large particles within the extract were appropriately filtered out using a muslin cloth followed by filtering with a Whatman No.1 filter paper. Consequently, the filtrate was concentrated initially by a rotary evaporator and after which made into dry powder by freeze-drying. While in a grinder, each fruit of *A. comosus* and *M. citrifolia* were chopped into smaller pieces and mixed. To get the fruit juices, the appropriate fruit waste was removed and filtered using a muslin cloth. The raw extracts were freeze-dried to produce a dry powder. All the powdered extracts were stored in an airtight container in a -20^{o}C freezer.

Milk Coagulation to Curd

To prepare 150 mg/mL of coagulant, 50 mg freeze-dried crude extracts of *M. indica*, *A. comosus* and *M. citrifolia* were combined in a 1:1:1 ratio. Next, 10 mL of 150 mg/mL coagulant were mixed with 20 mL of 10 % (w/v) skim milk solution (Difco, US). It was left to stand for 5 hours for completion of milk coagulation to curd.

Next, to obtain the curd alone, the whey was spun off in a centrifuge at rpm of 12 000 for 2 minutes. The curd obtained after discarding the whey was freeze-dried to become dry powder. *Mucor meihei* rennet was used to make a negative control curd (Sigma-Aldrich, US). The freeze-dried curds were stored in airtight containers in a -20^{o}C freezer.

Preparations of O. tenuiflorum and its Fortification of Curd

To obtain powdered dried *O. tenuiflorum*, the fresh leaves were cleaned and chopped into smaller pieces to be dried in an oven at 45^{o}C until dry from moisture. The dried material was ground to a powder. The powdered material (120 g) was soaked with 50% methanol and mix with a shaker for 48 hrs. To remove any large particulates from the *O. tenuiflorum* extract, it was firstly filtered with a muslin cloth followed by a Whatman No.1 filter paper. The extracted was concentrated with a rotary evaporator and then freeze-dried to yield dry powder.

The *O. tenuiflorum*, prepared curd and water were homogenized in the ratio of 1:2:10, respectively until properly mixed to obtain *O. tenuiflorum* enriched curd. While the sample of *Mucor meihei* rennet-formed curd was prepared similarly using curd formed from rennet and water at the ratio of 3:10, respectively.

Assay for DPPH Radical Scavenging

The DPPH radical scavenging test was performed using a slightly modified version of the prior technique.[13] Appropriate dilutions of a standard phenolic compound of gallic acid (Merck, US) and test samples of *O. tenuiflorum* extract, curd derived from selected plants, curd derived from selected plants + *O. tenuiflorum* and *Mucor meihei* rennet-formed curd were prepared by serial dilutions. For each sample, an equal volume of sample solution was mixed with 0.004 % (w/v) DPPH (Sigma-Aldrich, US) in ethanol (HmbG Chemicals, Germany) and incubated in the dark for 30 minutes. To read the absorbance a microplate reader was used at a wavelength of 517 nm. For the calculation of the DPPH radical scavenging activity of the samples, Equation 1 was used:

$$\text{Activity for DPPH Radical Scavenging } (\%) = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

The absorbance reading for the control without any test samples was $A_{control}$, while the absorbance reading with the sample was A_{sample}. The IC_{50} value, which shows the concentration of sample necessary to inhibit DPPH by 50%, was calculated by plotting the percentage of DPPH radical scavenging versus the concentration of the sample.

Test for Ferric Reducing Antioxidant Power (FRAP)

The test was conducted with some modifications to the previous method of FRAP assay.[14] To create a standard curve for the total phenolic content measurements, appropriate dilutions of the standard phenolic compound of gallic acid was produced in ethanol. The three test samples were generated in the same way by serial dilutions in water. To make a reaction mixture, 40 µL of sample solution were combined with 100 µL of 1% (w/v) potassium ferricyanide (Sigma-Aldrich, US) solution and 100 µL of 0.1 M potassium phosphate buffer (R&M Chemicals, UK), pH 6.6. For 20 minutes the reaction mixtures were incubated in a water bath at 50^{o}C. The reaction in the mixture was terminated by adding 100 µL of 10 % (w/v) trichloroacetic acid after it had been incubated. Then, in 200 µL of the reaction mixture, 40 µL of 0.1 % (w/v) ferric chloride (Sigma-Aldrich, US) and 200 µL of water were added and incubated at room temperature. In a microplate reader, the absorbance was measured at 700 nm. The findings were reported as gallic acid equivalent (GAE) in mg per gram by weight of the sample.

Test for Anti-diabetic Activity Using α-Amylase Inhibitory Activity

The *α*-amylase inhibitory activity test was performed using a slightly modified version of the prior technique.[15] Appropriate dilutions of standard *α*-amylase inhibitor acarbose (Glucobay 100 mg tablet, Bayer Inc., US) and the three test samples were prepared by serial dilution in water. To make the reaction mixture, 250 µL of a sample was combined with 50 µL of 2 U/mL *α*-amylase (Sigma-Aldrich, US) in 20 mM sodium phosphate buffer pH 6.9. The reaction mixture was incubated for 30 minutes. After that, 50 µL of 1 % (w/v) starch solution was added to the mixture as a substrate, and the mixture was incubated for 10 minutes at room temperature. The process was then stopped by adding 50 µL of 3,5-dinitrosalicyclic acid (DNS) reagent (Sigma-Aldrich, US) and cooled to room temperature after 5 minutes in an 85^{o}C water bath. In a microplate reader, the absorbance was measured at 540 nm. The sample's antidiabetic activity was determined using the following formula (Equation 2):

$$\alpha - \text{amylase inhibition activity } (\%) = \frac{\text{A control} - \text{A sample}}{\text{A control}} \times 100$$

The absorbance reading of the control without any test sample was $A_{control}$, while the absorbance reading of the sample was A_{sample}. To establish the IC_{50} value, a graph of the percentage of *α*-amylase inhibitory activity against sample concentration was generated.

Anti-bacterial Activity and Determination of Minimum Inhibitory Concentration (MIC)

A two-fold serial dilution of the initial concentration of 75 mg/mL was applied to determine the MIC value. A total volume of 25 µL of each concentration was transferred to the hole that was made on the Mueller-Hinton (Sigma-Aldrich, US) agar plate. Before that, the agar plate was spread uniformly using a sterile cotton swab with each type of bacteria grown to 1 x 10^{8} colony-forming units. The agar plate was inverted and incubated for 24 hours at 37^{o}C in the incubator. The agar plates were observed for obvious inhibition zones surrounding the holes. The MIC values were measured against two Gram-positive bacteria (*Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 11778) and three Gram-negative bacteria (*Acinetobacter baumannii* ATCC 19606, *Escherichia coli* ATCC 10534 and *Serratia marcescens* ATCC 13880).

Data Analysis

All the tests were done in quadruplicates and the results were presented as mean standard deviation. The data was graphically plotted using the Microsoft Excel 2019 software. The IC_{50} value was determined using GraphPad Prism 8 software program.

Results and Discussion

Evaluation of Antioxidant Activities

The DPPH test is a spectrophotometric approach based on the quenching of stable colored radicals (DPPH) that shows the antioxidants' radical scavenging activity even when present in complex biological mixtures like plant or food extract.[16] The antioxidant activity according to DPPH test is as shown in Table 1 and can be ranked as follows: gallic acid >curd derived from selected plants + *O. tenuiflorum*>*O. tenuiflorum*> curd derived from selected plants >*Mucor meihei* rennet formed curd. The IC_{50} values in this study were calculated from graphs found in Figure 1. A study showed that the IC_{50} value for gallic acid was 0.006mg/ ml.[17]

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Table 1: The IC_{50} values determined by DPPH test

Sample	IC_{50} mg/mL ± S.D.
Gallic acid	0.01 ± 0.30
Curd derived from selected plants + *O. tenuiflorum*	1.47 ± 3.82
O. tenuiflorum extract	4.45 ± 5.38
Curd derived from selected plants	8.49 ± 10.56
Mucor meihei rennet formed curd	-

The replicates in the experiment were done in quadruplicate. The data were presented as a mean ± standard deviation (S.D).

Table 2: The total phenolic content of the samples determined by FRAP assay

Sample	Total Phenolic Content (mg GAE/g) ± S.D.
O. tenuiflorum extract	399.6 ± 0.1
Curd derived from selected plants + *O. tenuiflorum*	370.8 ± 0.3
Curd derived from selected plants	333.3 ± 0.2
Mucor meihei rennet formed curd	-

The replicates in the experiment were done in quadruplicate. The data was presented as a mean ± standard deviation (S.D.)

This showed that gallic acid is a potent antioxidant agent as also indicated in this study. The IC_{50} value of radical scavenging activity of the *O. tenuiflorum* extract shows was a dose-dependent inhibition and previously identified with important phytochemicals for antioxidant and anti-inflammatory activities.[18,19] The radical scavenging activity of the curd derived from selected plants fortified with *O.tenuilflorum* was found to be better than the curd derived from the selected plants alone thereby *O. tenuilflorum* improved the antioxidant outcome of the curd.

The FRAP antioxidant properties were assessed using gallic acid standard which yielded the standard curve shown in Figure 2. Table 2 shows that the total phenolic content is highest for *O.tenuiflorum*> curd derived from selected plants + *O. tenuiflorum*> curd derived from selected plants >*Mucor meihei* rennet formed curd. In this assay, *O. tenuiflorum* showed the highest phenolic content. The phenolic compounds included eugenol, cirsilineol, isothymusin, isothymonin, rosmarinic acid, orientin, vicenin and others which makes this plant a good antioxidant.[20,21] The extract of *O. tenuiflorum* was found to contain about 51 mg GAE/g of total phenolic content.[22] Another data revealed that the total phenolic content for *O. tenuiflorum* was about 365 mg GAE/g.[23] The antioxidant ability of *M. indica, A. comosus*, and *M. citrifolia* has been reported in several literature data including their total phenolic contents. For instance, *M. indica* has been shown with total phenolic content of about 91 mg GAE/g.[24] Furthermore, in a study, the pulp of *M. citrifolia* was to have a total phenolic content of about 80 mg GAE/g of dry weight.[25] It appeared the antioxidant activity within the fortified curd is not provided by *O. tenuiflorum* alone but likely as well contributed by the three milk coagulant plants.

Evaluation of α-Amylase Inhibition Assay

The IC_{50} values for various samples was estimated using the graphs of % inhibition of α-amylase versus concentration used as shown in Figure 3. The effectiveness of *α*-amylase inhibition by various samples is summarized in Table 3 and ranked as follows: curd derived from selected plants + *O. tenuiflorum*> curd derived from selected plants >*O. tenuiflorum*> Acarbose >*Mucor meihei* rennet formed curd. The samples tested were having almost similar IC_{50} values to acarbose, a standard inhibitor compound in this assay.

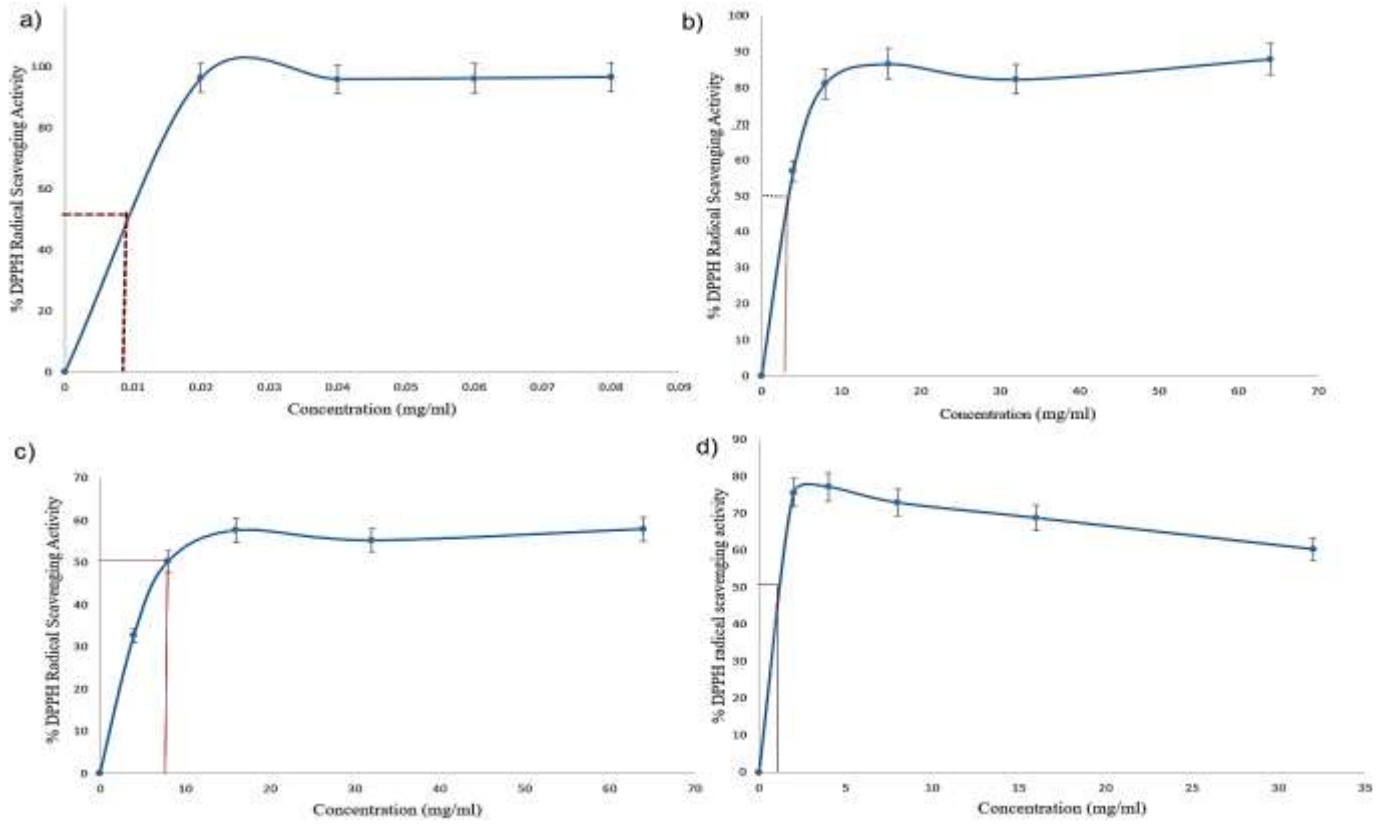

Figure 1: The percentage of DPPH scavenging activity of gallic acid is represented as graph a); *O. tenuiflorum extract*, b); curd derived from selected plants, c) and curd derived from selected plants + *O. tenuiflorum*, d)

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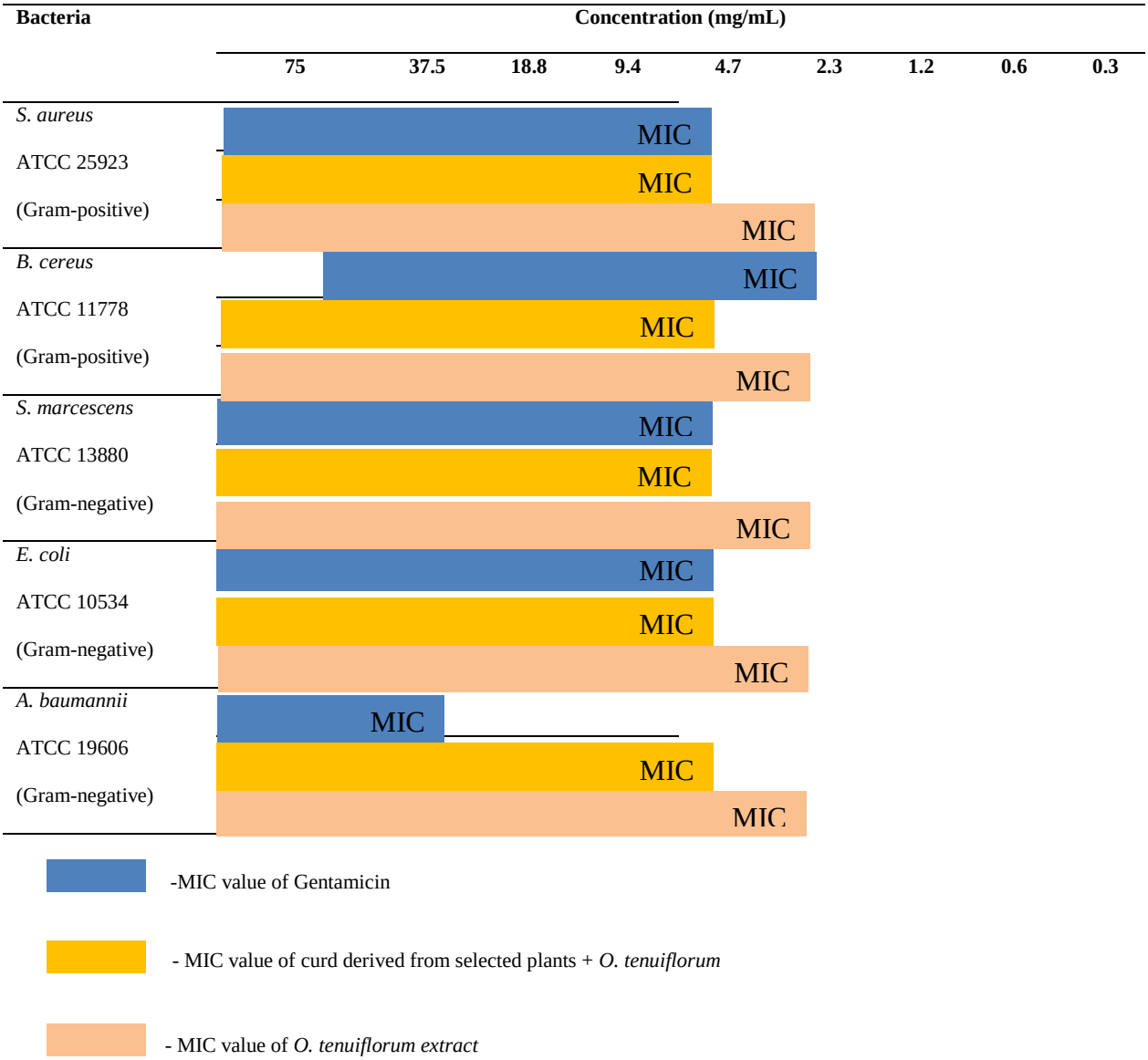
Table 3: The IC_{50} values for α-amylase inhibitory activity of various samples

Sample	IC_{50} mg/mL ± S.D.
Curd derived from selected plants + *O. tenuiflorum*	3.19 ± 1.59
Curd derived from selected plants	3.96 ± 1.96
O. tenuiflorum extract	4.51 ± 4.56
Acarbose	4.56 ± 7.05
Mucor meihei rennet formed curd	-

The replicates in the experiment were done in quadruplicate. The data were presented as a mean ± standard deviation (S.D).

Together with other critical illnesses, diabetes is common and on the rise. Due to the significant adverse effects of commercially available anti-diabetic medicines, new treatment methods are being researched to control postprandial glucose levels.[26] Amylase inhibitors are antidiabetic medications, the most common of which being acarbose. These medicines have a significant benefit and are effective in the treatment of non-insulin-dependent diabetic mellitus type 2 diabetes.[27] Acarbose has been explored in the treatment of type 2 diabetes.[28] Long-term acarbose therapy has been shown to reduce the risk of diabetes, hypertension, and cardiovascular disease. It was also mentioned that acarbose has shown its relative potency as an α-amylase inhibitor.[29] Yilmazer-Musa et al reported the acarbose used in their study recorded an IC_{50} value of 6.9±0.8 µg/mL.[30] The variance in the IC_{50} value reported in the present study is due to the difference in grade and purity of the acarbose (Glucobay 100 mg tablet manufactured by Bayer Inc., US) whereby a pharmaceutical grade drug contains non-medicinal ingredient or excipient as well. The IC_{50} value for *O. tenuiflorum* showed that it has anti-diabetic properties. Leaves of *O. tenuiflorum* have been used to treat diabetes and its complications.[31] Those who used *O. tenuiflorum* in addition to their regular medicines experienced significant improvements in glucose management.

Table 4: Comparison of the MIC values for various samples against gram-positive and gram-negative bacteria



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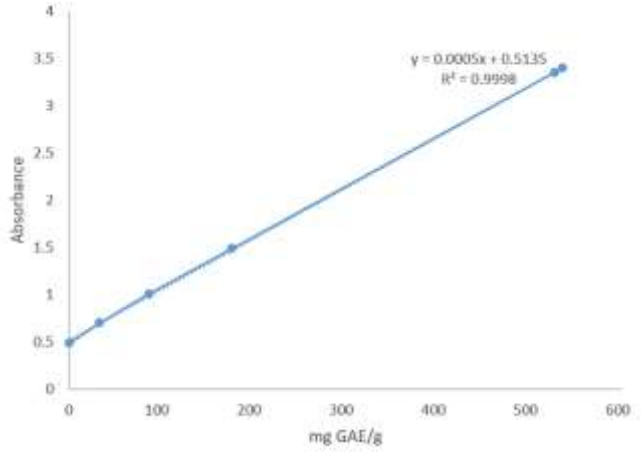

Figure 2: The gallic acid standard calibration curve for total phenolic content quantification.

In a 90 days clinical trial involving *O. tenuiflorum* (or also known as *Ocimum sanctum*) given together with Glibenclamide (a common diabetic drug) has the outcome shown to have significantly lower fasting blood glucose levels than dosing the diabetic mellitus type 2 patients with the latter alone.[32]

The curd derived from selected plants in this study contains a mixture of three plants of *M. indica, A. comosus, and M. citrofolia* and found with relatively similar IC_{50} value to that of acarbose in inhibiting α-amylase. These plants therefore potentially having anti-diabetic activities of their own. Among these *M. indica* exhibited dose-dependent inhibition of α-amylase and α-glucosidase activities.[33,34]. Noticeably the IC_{50} value improved to 3.19±1.59 after been fortified with *O. tenuiflorum* extract (Table 3). Therefore, the use of multiple plants with known antidiabetic ability may have induced synergistic action in lowering the IC_{50} value calculated.

Minimum Inhibitory Concentration (MICs)

The MIC values of the three samples can be concluded in the order of descending strength of *O. tenuiflorum extract*>curd derived from selected plants + *O. tenuiflorum* ≥ gentamicin for the bacteria tested. The MIC values were determined by observing for the hole having the lowest concentration of the sample capable to inhibit growth of the tested bacteria (as shown in Figure 4).

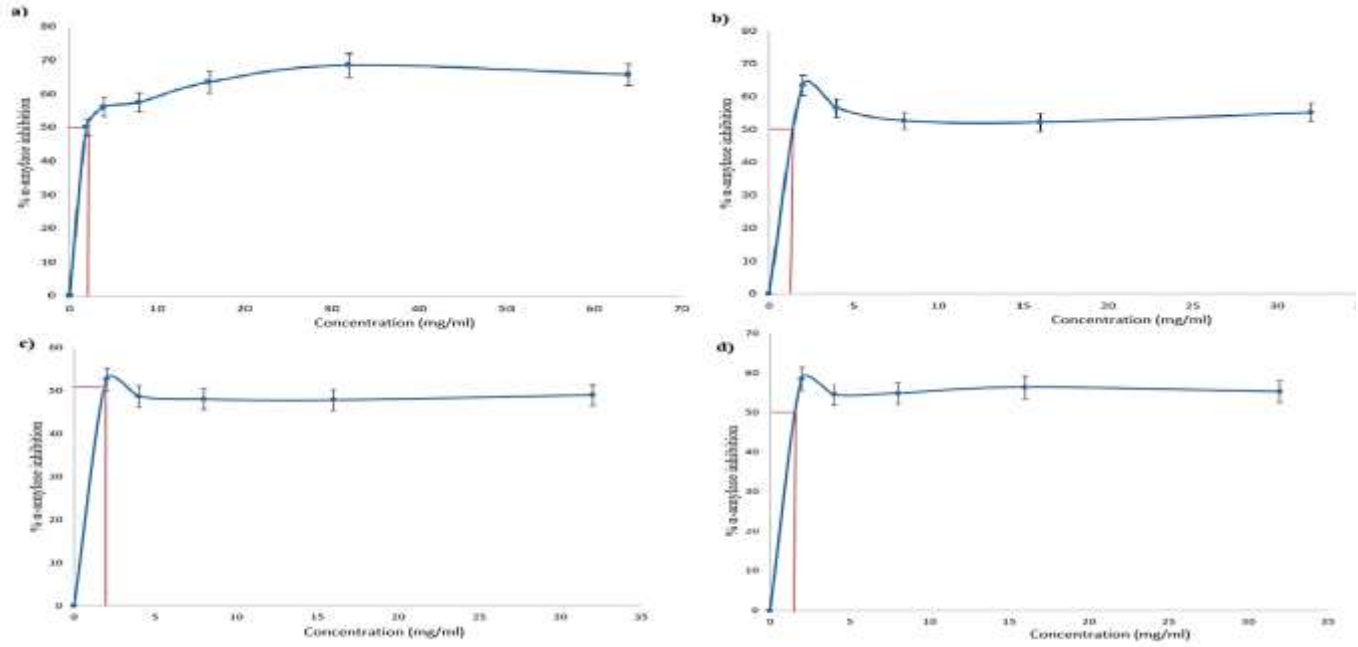

Figure 3: The α-amylase % of inhibition for gallic acid represented as graph a); *O. tenuiflorum* extract, b); curd derived from selected plants, c) and curd derived from selected plants + *O. tenuiflorum*, d)

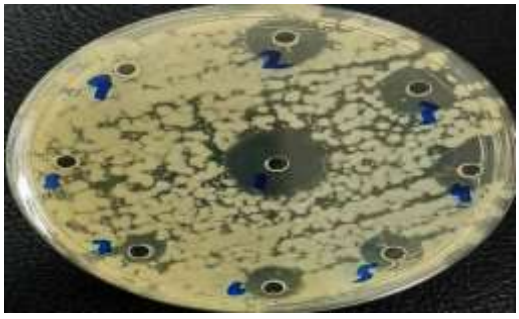

Figure 4: MIC determination for curd derived from selected plants + *O. tenuiflorum* against *S. aureus* with the centre hole representing the initial concentration of 75 mg/mL.

Based on the comparison of all three MIC values displayed in Table 4, the *O. tenuiflorum extract* had the best anti-bacterial activity while the curd derived from selected plants + *O. tenuiflorum* has similar or better activity than the gentamicin outcomes. The *O. tenuiflorum* extract shows the lowest MIC values compare to other samples, which is 1.2 mg/mL. Even though, gentamicin is an antibiotic containing a pure substance, *O. tenuiflorum extract* interestingly showed lower MIC value than gentamicin. It is no surprise as scientific data already established the potency of *O. tenuiflorum* against pathogens.[35,36]

Conclusion

The overall results showed the plant coagulants derived curd fortified with *O. tenuiflorum* exhibited higher potency for all biological activities tested compared to plant-derived curd alone. This indicated that *O. tenuiflorum*, selected based on its medicinal potentials successfully demonstrated its antioxidant, antidiabetic and antibacterial

activities. Additionally, the three plants of *M. indica*, *A. comosus* and *M. citrifolia* while useful for coagulating the milk to curd, also possess phytochemicals acting synergistically with *O. tenuiflorum* toward improving the activities of the curd formed by them. *Mucor meihei* rennet-formed curd was without any relevant active phytochemicals and evidently showed no apparent biological activities. The findings in this study is useful in the development of functional dairy food for vegetarians and free of cruelty towards animals. Potentially attempts can be made to isolate the active compounds with biological activities within *O. tenuiflorum* or other natural products and include them in the curd. Many such attempts to isolate the important active compounds steadfastly being achieved from natural products.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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