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Antimicrobial, Antioxidant and Anti-inflammatory Effects of Cocoa Bean Husk (CBH) Extract

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Abstract

Plants are an excellent source of naturally occurring bioactive compounds that have shown promising results in the medical and pharmaceutical fields. In this study, cocoa bean husk (CBH) ethanolic extract showed potent inhibitory action. CBH extract demonstrated broad-range antibacterial potential against positive Gram and negative Gram bacteria, which are resistant to many drugs, with inhibition zones ranging from 14.7 to 21.0 mm. CBH extract demonstrated antifungal properties against *A. fumigatus*, *A. niger*, and *C. albicans*, displaying inhibition zones of 28.7, 31.0, and 18.7 mm, respectively. The MIC values of CBH ranged from 31.2 to 250 µg/mL. CBH extract exhibited strong antioxidant and anti-inflammatory effects. CBH extract has no cytotoxic effects on normal cells. Additionally, (+)-catechin was found to be the main bioactive polyphenolic component by HPLC analysis (13.36 mg/g), which may be the cause of the CBH extract's biological activity.

Keywords: Cocoa bean husk (CBH); Antimicrobial; Antioxidant; Anti-inflammatory, Polyphenolic compounds

1. Introduction

A large number of bioactive metabolites originate from plants. Furthermore, it has been demonstrated that a wide variety of biomolecules are produced by plants. Within the family Sterculiaceae, *Theobroma cacao* Linn (*T. cacao*) is a species of tropical tree. Adi-Dako [1] stated that a significant quantity of waste is produced in the form of cacao husks (CH) during the process of recovering the fruit's beans, which provide the main commercial component of cacao. The hues of cacao husks range typically from maroon to green and *T. cacao* is the primary component of chocolate and various derivative products like butter, cake, powder, and cocoa liquor. Cacao husks have been used as fertilizer, animal feed, and as an initial base for the synthesis of activated carbon [2]. Moreover, cocoa husks might be a great source of foods high in dietary fiber that can dramatically lower the chance of developing many diseases caused by free radicals [3]. The cocoa husk bean and its derivatives are extremely helpful to health, and numerous anti-hypercholesterolemia supplements are made from these husks [4]. Several researches have suggested the using of cocoa husks as alternatives to a substitute for production of effective biological products [5]. According to Martinez *et al*. [6], CHB is regarded as a source of organic chemicals with potential applications in medicine and it contains high amounts of biologically active flavonoids and fiber that could be used for

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pharmaceutical manufacturing [7]. The health benefits of CBH have been well studied recently, since it contains antimicrobial compounds such as steroids, glycosides, alkaloids, flavonoids, and saponins [8,9]. *Theobroma cacao* L. is linked to a high range of biological characteristics, and it is a rich source of phytochemicals, with polyphenols constituting one of the largest classes of substances. However, cacao (*T. cacao*) has strong antibacterial qualities in addition to increased anti-inflammatory and antioxidant activity [10]. A variety of human diseases, including dyspepsia, circulatory issues, nervous system disorders, heart problems, and bacterial infectious diseases, can be prevented and treated with CBHs [11]. CBH is regarded as an agro-industrial residue, and it is the primary cocoa industry byproduct. Every year, Mexico for example produces over 27 thousand tons of dried cocoa beans, and 3240 tons of wastes from CBH are produced, assuming that the CBH accounts for 12% of the weight of the beans [12]. Certain artisanal CBH uses can be found in Mexico's cocoa-producing regions, such as in the baking of cookies and the sporadically produced bokashi, an organic fertilizer for local usage. But husks are thrown away as waste in the great majority of cases. Because of its high level of fiber and antimicrobial properties, CBH is an intriguing new component in the food economy. It may also be an important source of biologically active chemicals such as theobromine and phenols [13]. Other research has shown that some of the properties of CBH, such as its high content of phenolic components and approximately 18.2 mg/g of dietary fiber, have potential applications in the field of nutritious foods [14], including protein (170 mg/g), high content of linoleic acid, excellent food-grade fat (67 mg/g), lignin, pectin, and cellulose [15]. p-hydroxybenzoic acid and epicatechin are among the phenolic chemicals found in CBH methanol extracts, and the substance has a theobromine concentration of about 12.9 mg/g [14]. The cotyledons of cocoa seeds have been shown to retain phenolic chemicals. However, during fermentation, significant quantities of these chemicals diffuse out of the cotyledon. Indeed, upon fermentation, a portion of the phenols present in the cotyledon is transferred to the husk, converting CBH into a material abundant in bioactive substances. Because of its lignocellulosic structure, CBH has been shown in recent research to have prospective applications in the construction of carbon-activated monoliths [16]. The investigation aimed to examine bioactive constitutes of the methanolic extract of CBH and to evaluate their antibacterial, anti-inflammatory, and antioxidant.

2. Experimental (Materials and Methods)

Ethical statement:

Cairo University's Institutional Animal Care and Use Committee approved the current protocol (IACUC number: 08072023664)**.**

2.1. Extraction of cocoa been husk

The cocoa pod husk was cleaned, chopped into little pieces, and then baked at 50ºC for two consecutive days. It was then blended into a powder and sieved to produce a fine, homogenous powder. About 150 grams of cacao pods in total were placed in a glass beaker, and 600 milliliters of 70% ethanol solvent was added. After that, the mix was homogenized for 10 minutes at a speed of 70 rpm using an ultrasonic homogenizer. A rotating vacuum evaporator is used to concentrate the mixture after vacuum filtration separates it from the residue [17].

2.2. Antimicrobial potential

2.2.1. Microorganisms

Ten clinical pathogenic bacterial isolates have been used in this study including the Gram +ve bacteria (*Streptococcus pyogenes*, *Enterococcus faecalis*, and *Staphylococcus aureus*) and Gram –ve bacteria (*Klebsiella pneumoniea*, *Acinetobacter baumannii*, *Escherichia coli*, *Proteus vulgaris*, *Proteus mirabilis*, *Serratia marcescens*, and *Pseudomonas aeruginosa*). All bacterial isolates were obtained from Kasr AL Ainy Hospitals and were identified using BioMérieux ®, Inc.'s (Hazelwood, MO, USA) conventional VITEK 2 compact 15 system.

Three pathogenic fungal isolates were used including *Candida albicans*, *Aspergillus fumigates*, and *Aspergillus niger*, obtained from Kasr AL Ainy Hospitals.

2.2.2. Antibiotics screening test

The antibiotics screening test was carried out by the conventional Kirby-Bauer disc diffusion method for all bacterial strains in accordance with the guidelines of [18]. The antibiotic discs (Oxoid, Ltd. Co, UK) used for negative and positive gram bacteria were: Ofloxacin (5 µg), Cefoxitin (30 µg), Cefazolin (30 µg), Doxycycline (5 µg), Tetracycline (30 µg), Levofloxacin (5 µg), Meropenem (10 µg), Imipenem (10 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Ceftazidime (30 µg), Cefotaxime (10 µg), Cefepime (30 µg), Tigecyclinee (15 µg), Ampicillin/Sulbactam (30 µg), Amoxicillin/clavulanic acid (30 µg), and Amikacin (30 µg).

2.2.3. Screening for Antibacterial Activity

Using the disc-diffusion technique [19], the antibacterial potency of CBH extract was evaluated against the ten tested microorganisms. 6 mm Whatman discs were impregnated by 25 µL of 50 mg/ml ethanolic extract of cocoa bean husk (CBH) and then seeded with 100 µl tested bacteria (10^8 CFU). The suspension was adjusted using 0.5 McFarland standards [20]. The discs were then placed on Mueller-Hinton sterile agar plates. Amikacin disc served as a positive control. The seeded plates were allowed to dry for 15 minutes. All Plates were incubated for eighteen hours at 37°C. Following the period of incubation, the inhibitory zone was determined using a digital caliper. Triplicate tests were performed, and the average value (± standard deviation) of the results was recorded.

2.2.4. Minimum inhibitory concentrations test of CBH with bacterial isolates

Using a 96-well microtitre plate, the MIC was measured according to the guidelines provided by the [21] microdilution method. In the plate wells, 100µL of extracts that had been diluted two times in Muller-Hinton broth were added. Following that, extracts containing the test compounds were put into every well until a 200 µL ultimate volume was reached, except a blank column used as a control. Inoculum was standardized at 0.5 Macfarland. The compounds under test had quantities with a range from 500 to 3.9 µg/ml. At 37°C, for 24 hours, the plates were incubated. The lowest concentration that prevented bacteria from growing was found to be the MIC, while turbidity acted as an indicator of growth.

2.2.5. Antifungal properties of Cocoa bean husk (CBH) ethanol extract

The antifungal activities of CHPE were tested against pathogenic fungal isolates and yeast (*Candida albicans*, *Aspergillus fumigates*, *Aspergillus niger*). The diffusion disc method was applied according to [22]. A sterile rod display was used to disseminate 100µl of the spore suspension (10^8 spores/ml) of the investigated fungi across the solidified potato dextrose agar (PDA) plates. After being saturated with 10µl of CHPE extract, sterile filter discs (Whatman no. 1, England, 6 cm diameter) were put on the agar surface. Discs of fluconazole were used as a positive control. Using a digital caliper, the mean diameter of the inhibitory zones on the plates was measured after they were incubated for four to five days at 28 0C. Every test was run via three replications. The mean value (± standard deviation) of the results is displayed.

2.2.6. Minimum inhibitory concentrations (MIC) determination of CBH with fungal isolates

On 96-well microdilution plates, the MIC of cocoa bean husk (CBH) ethanol extract was obtained. Following established procedures [23], 10 µl of a spore suspension (10^8 spores/ml) was utilized as the inoculum. Furthermore, growth and non-growth controls were generated. The microplates were incubated at 25°C for 72 hours. When there was no turbidity in the well, MIC values were determined.

2.3. In vitro antioxidant effects of CBH extract

[24] Stated that the stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was used to compare the free radical scavenging abilities of cocoa bean husk (CBH) extract with standard vitamin C. In summary, 1.0 mL of Cocoa bean husk (CBH) extract at 1.0 mL of DPPH (1.0 M in methanol), several concentrations (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, and 0.19 g/mL) were centrifuged and allowed at the dark for thirty minutes in ambient temperature. Utilizing a UV-Vis spectrophotometer (AU-2701, Systronics), the absorbance at 517 nm was obtained. Chemicals that were absent from the sample and DPPH were used as a control. A methanol blank solution was employed. The following formula was used to get the percentage of inhibition.

Scavenging percentage = (Pc − Ps)/Pc × 100.

Where Pc= vitamin C absorbance, Ps = Cocoa bean husk (CBH) extract.

2.4. In vitro anti-inflammatory activity

2.4.1. Erythrocyte suspension preparation

According to [25], participants in good health provided three milliliters of fresh whole blood, which was put into heparinized tubes after a ten-minute centrifugation at 3000 rpm. The volume of normal saline utilized to dissolve the pellet of red blood was the same as the supernatant's volume. A 40% v/v suspension was created utilizing a buffer solution that is isotonic (7.4 pH, 10 mM sodium phosphate buffer). once the dissolved red blood pellets volume was known. The buffer solution contains 9 g of NaCl, 0.2 g of NaH2PO4, and 1.15 g of Na2HPO4 in one liter of distilled water. This involved applying reconstituted red blood cells, or re-suspended supernatant.

2.4.2. Hypotonicity induced hemolysis

Following the procedure described by [25] samples of the extract were dissolved in distilled water (hypotonic solution). The hypotonic solution (5 ml) was used to create duplicate sets of centrifuge tubes (per dosage) with graduated extract dosages (100–1000 µg/mL). 200 g/mL of indomethacin and 5 mL of the vehicle—distilled water were both present in the control tubes. The erythrocyte suspension (0.1 mL) was added to each tube and gently mixed. Following an hour incubation at ambient temperature (37 °C), the mixtures were centrifuged at 1300 g for three minutes. Milton Roy (Spectronic) spectrophotometer was used to measure at 540 nm, the amount of absorption (OD), which indicates the hemoglobin concentration of the supernatant. The hemolysis percentage was determined under the supposition that in the presence of distilled water, 100% hemolysis is produced. The following formula was used to determine the extract's percentage reduction of hemolysis:

% Prevention of haemolysis = 1- ((OD2-OD1)/ (OD3-OD1)) *100

According to [25], the absorbance of the test sample in an isotonic solution (OD1), the test sample's absorbance in a hypotonic solution (OD2), and the absorbance of the control sample in a hypotonic solution (OD3).

2.5. In vitro determination of cytotoxicity

The cytotoxicity of the Cocoa bean husk (CBH) extract was evaluated using human lung fibroblast cell line (WI-38 cell). To generate a complete monolayer sheet, the 96-well tissue culture plate was filled with one X 105 cells/ml (100 µL) and incubated at 37 °C for 24 hours. Once a combined sheet of cells had grown, the microtiter 96-well plate growth substance was discarded, and the monolayer cell was then washed twice using the washing media. Two percent serum was added to RPMI medium to dilute the test sample twice (maintenance medium). Each dilution was examined in individual wells with 0.1 ml, leaving 3 wells served as controls that only received maintenance medium. After being incubated at 37°C, the plate was examined. Visible signs of toxicity, as shrinkage, rounding, granulation, or complete or partial disappearance of the monolayer, were checked in the cells. Then, a 5 mg/ml methyl-thiazolyl tetrazolium (MTT) solution was prepared (bio basic Canada Inc). Next, each well received 20 uL of the MTT solution. Shake hard at 150 rpm for 5 minutes to completely incorporate the MTT into the media. Let the MTT digest in an incubator at 37 °C with 5% CO2 for 4 hours. Formazan (MTT metabolic product) was reconstituted in 200 uL of dimethyl sulfoxide (DMSO). Put the mixture on a shaking table at 150 rpm and shake for five minutes to ensure that the solvent and formazan are well mixed. The number of cells following background removal at 620 nanometers and the read at 560 nm need to be directly correlated [26]. According to [27], the morphological examination is associated with both cell viability and extensive morphological modifications that occur at the cell surface or in the cytoskeleton.

2.6. HPLC Analysis of total polyphenolic components

Using the HPLC technique with a (PDA) photo-diode array detector, the phenolic components in cocoa husk were

identified and quantified, following the guidelines provided by [28]. Five milliliters of 70% methanol were used to extract the bioactive components. The mixture was then ultrasonicated for thirty minutes and then centrifuged for ten minutes at 3000 rpm. The process was repeated after collecting the supernatant. The obtained supernatants were mixed in a flask, and 70% methanol was added to adjust the ultimate volume (10 mL). The extracts were passed through a 0.45 µm nylon membrane filter, Chromafil Xtra (Germany, Machery-Nagel) before injection. The Shimadzu liquid chromatography, which included SIL-10AF autosampler, SPD-M20A photodiode array detector, Shimadzu CTO-20AC column oven, and LC-20AD solvent supply module, was used to conduct the HPLC analysis. The software Lab Solution Lite (Release 5.52) was used to support the instrument. Gradient elution was used to separate the bioactive components on Inertsil ODS-3V (5 µm particle size, GL Sciences, 250 mm × 4.6 mm) at a flow level of 0.8 mL/min using a mixture of HPLC grade methanol (solvent A) and (solvent B) formic acid1%. The next was the conditions for gradient elution. In the mobile phase, the solvent content was 10% initially, rising linearly to 32% A at 15 minutes, 40% A at 20 to 25 minutes, and 60% A at 30 minutes. A 30 ◦C temperature was maintained for the column and detector, and a 20 µL injection volume was used. Chromatograms were obtained at a 280 nm wavelength. The quantification process was carried out using an external calibration method, and the identification of separated components was accomplished by comparing the retention durations and UV spectra using materials that serve as references.

3. Analysis of statistical data

The results were presented as mean ± SEM. The data were analyzed using statistical software SPSS version 20.0. A simple one-way ANOVA was used to examine the effect of week within treatments and the effect of treatment within weeks. Duncan's Multiple Range test was used to distinguish between significant means at $P<0.05$. Univariate two-way ANOVA model (4 treatments ×10 weeks). A student t-test was used to compare the fertility parameters of the control and the selected Maca treatment. The significance level was set at $P<0.05$

4. Results and discussion

4.1. Antibiotic susceptibility pattern of bacteria

Table (1) represented the antibiotic susceptibility pattern of bacterial strains. *S.auraus* found resistance to amoxicillin/clavulanicacid, ampicillin/sulbactam, cefotaxime, ceftazidime, ciprofloxacin, doxycycline, gentamicin, levofloxacin, and ofloxacin, these results are in the agreement with El-Kattan *et al*. [29]. The resistance mechanism of *S. aureus* may be due to genetic factors and mutations [30]. *S. aureus* is a prevalent bacterial pathogen that is resistant to multiple drugs and can cause a variety of diseases [31]. On the other hand, *S.auraus* were sensitive to amikacin, cefoxitin, cefazolin, cefepime, imipenem, meropenem, tetracycline, and tigecycline. *E.* faecalis were also demonstrated a resistance with cefotaxime, cefazolin, cefepime, cefoxitin, ceftazidime, ciprofloxacin, levofloxacin, gentamicin and ofloxacin, which was similar to those reported previously [32]. Pieniz *et al*. [33] stated that, because *E. faecalis* can acquire up and spread antibiotic resistance genes, it exhibits multidrug resistance. Hurst *et al*. [34] showed that, *S. pyogenes* are responsible for several clinical conditions, and it was resistant tocefepime, ampicillin/sulbactam, cefazolin, cefotaxime, amoxicillin/clavulanic acid, cefoxitin, ciprofloxacin, doxycycline, and gentamicin [35]. In this study *A. baumannii* showed resistance to cefazolin, cefepime, cefotaxime, ceftazidime, ciprofloxacin, doxycycline, cefoxitin, gentamicin, ofloxacin, and levofloxacin, These findings were not in line with the results published by Guckan *et al*. [36], but those findings were in agreement with Musyoki *et al*. [37]. Mostly *E. coli* was resistant to cefazolin, cefotaxime, ampicillin/sulbactam, ciprofloxacin, ceftazidime, doxycycline, amoxicillin/clavulanic acid, levofloxacin, and tetracycline; this was comparable to earlier research [38]. One well-known nosocomial infection that has been linked to multidrug resistance (MDR) and pan drug resistance in recent years is *K. pneumoniae* [39]. *K. pneumoniea* exhibited a strong resistance against cefazolin, cefepime, cefotaxime, gentamicin, ofloxacin, ampicillin/sulbactam, ceftazidime, cefoxitin, ciprofloxacin, amoxicillin/clavulanic acid, levofloxacin, and tigecyclinee, this rise of antibiotic resistance in *K. pneumoniae* was previously documented [29]. According to Hasan *et al*. [40], the virulence

factors of *P. mirabilis* can lead to various nosocomial and opportunistic infectious diseases. Comparable to the findings of the research published previously [41] *P.mirabilis* showing different levels of resistance to antibiotics, cefazolin, amoxicillin/clavulanic acid, doxycycline cefotaxime, tetracycline, and ciprofloxacin. Furthermore, *Pr. Vulgaris* showed resistance to cefazolin, cefepime, ceftazidime, ciprofloxacin, cefoxitin, gentamicin, and ofloxacin, this finding was in contrast to Kassam *et al.* [42] but was in agreement with Talebi *et al.* [43]. *P.aeruginosa* isolates displayed resistance against ofloxacin, ceftazidime, cefotaxime, ciprofloxacin, doxycycline, cefoxitin, ampicillin/sulbactam, cefazolin, gentamicin, amoxicillin/clavulanic acid, cefepime, tetracycline, levofloxacin, imipenem, and meropenem, these findings correspond with those of Bazaid *et al.* [44]. According to Imanah *et al.* [45], *P. aeruginosa* can readily acquire antibiotic resistance and has become resistant to a variety of drugs. Furthermore, the increasing rate of MDR *P. aeruginosa* in many countries poses a serious therapeutic problem [46]. Nosocomial infections have been linked to *S. marcescens*, an opportunistic pathogen [47]. *S. marcescens* exhibited resistance to ofloxacin, amoxicillin/clavulanic acid, cefazolin, ampicillin/sulbactam, cefepime, cefotaxime, cefoxitin, tetracycline, and ceftazidime, similar results stated by Zivkovic Zaric *et al*. [48]. On the other hand, all tested bacteria were sensitive to amikacin; however, tigecycline, meropenem, and imipenem were successful in combating most tested strains, these findings were consistent with previous studies [38, 44]. Negative and positive gram bacteria are still susceptible to carbapenems [49].

Table 1: Antibiotic susceptibility pattern of pathogenic bacterial strains

Antibiotic discs	***S. aureus***	***E. faecalis***	***S. pyogenes***	***A. baumannii***	***E. coli***	***K. pneumoniea***	***Pr. vulgaris***	***Pr. mirabilis***	***P. aeruginosa***	***S. marcescens***
Amoxicillin/Clavulanic Acid (30 µg)	R	I	R	S	R	R	I	R	R	R
Amikacin (30 µg)	S	S	S	S	S	S	S	S	S	S
Ampicillin/Sulbactam(30 µg)	R	S	R	I	R	R	S	S	R	R
Cefazolin (30 µg)	S	R	R	R	R	R	R	R	R	R
Cefepime (30 µg)	S	R	R	R	I	R	R	S	R	R
Cefotaxime (10 µg)	R	R	R	R	R	R	I	R	R	R
Ceftazidime (30µg)	R	R	S	R	R	R	R	S	R	R
Cefoxitin (30 µg)	S	R	I	R	S	R	R	S	R	R
Ciprofloxacin (5 µg)	R	R	R	R	R	R	R	R	R	S
Doxycycline (5 µg)	R	S	R	R	R	I	S	R	R	S
Gentamicin (10 µg)	R	R	R	R	S	R	R	S	R	S
Imipenem (10 µg)	S	S	S	S	S	S	S	I	R	S
Meropenem (10 µg)	S	S	S	S	S	S	S	S	R	S
Levofloxacin(5 µg)	R	R	S	R	R	R	S	S	R	S
Ofloxacin (5 µg)	R	R	S	R	I	R	R	S	R	R
Tetracycline (30 µg)	S	I	S	I	R	I	I	R	R	R
Tigecyclinee (15 µg)	S	S	S	S	S	R	S	S	S	S

Note: Resistant (R), Intermediate (I), Sensitive (S).

Following the overuse of antibiotics, pathogenic bacteria are becoming more adept in generating and dispersing antimicrobial resistance. Researchers discovered antibiotic substitutes that are less expensive than antibiotics while yet having good efficiency and minimal human harm. Since ancient times, plant extracts-whether they are alcoholic or aquatic-have been used. According to Rahayu et al. [17], multiple studies have documented the antibacterial effectiveness of CBH extract [50, 51]. Ten bacterial isolates and three fungal isolates (clinical specimens) were used in the current investigation. Table (2) lists the bacterial isolates that demonstrated multidrug resistance ability. Significant activity was observed against Gram-negative and Gram-positive bacteria with the 70% ethanolic extract of CBH (Figures 1 and 2). Against P. aeruginosa, And Gram-

positive bacteria with the 70% ethanolic extract of CBH (Figures 1 and 2). Against *P. aeruginosa*, the strongest inhibitory action was found 21.0 mm, followed by *S. aureus*, *S. marcescens*, *P. mirabilis*, *S. pyogenes*, *K. pneumoniea*, *E.faecalis*, *A. baumannii*, *E. coli* and *P. vulgaris* with inhibition zones reached 18.0, 18.0, 17.3, 16.7, 16.7, 15.7, 15.7, 15.0, 14.7 mm respectively. These findings are consistent to the previous reports [52,53,54,55]. Numerous studies have noted the effectiveness of CBH extract due to chemical compounds, such as bio-active materials, procyanidins, flavonoids, polyphenols, catechin, and epicatechin, additionally, it contains the tricyclic chemical structure of flavonoids, which controls their antioxidant properties and hunt down reactive oxygen species [17, 56]. Additional studies confirmed that cacao showed superior efficacy against specific pathogenic microorganisms [57].

Table 2: Antibacterial activity of Cocoa bean husk (CBH) ethanol extract against pathogenic bacterial strains

Bacteria	**Inhibitory zone in mm (Mean ± SD)**	
	CBH Ethanol Extract	**Positive Control (Amikacin)**
Staphylococcus aureus	18.0 ± 0.82	18.0 ± 0.82
Enterococcus faecalis	15.7 ± 0.94	17.3 ± 0.47
Streptococcus pyogenes	16.7 ± 0.47	20.0 ± 0.82
Acinetobacter baumannii	15.7 ± 0.47	20.0 ± 0.82
Escherichia coli	15.0 ± 0.82	16.7 ± 0.47
Klebsiella pneumoniea	16.7 ± 0.94	17.3 ± 0.47
Proteus mirabilis	17.3 ± 0.47	20.0 ± 0.82
Proteus vulgaris	14.7 ± 0.47	20.0 ± 0.82
Pseudomonas aeruginosa	21.0 ± 0.82	17.7 ± 0.47
Serratia marcescens	18.0 ± 0.82	18.0 ± 0.82

One-way ANOVA was used for statistical analysis, and significance ($P<0.05$) was assessed using Duncan's multiple comparison tests.

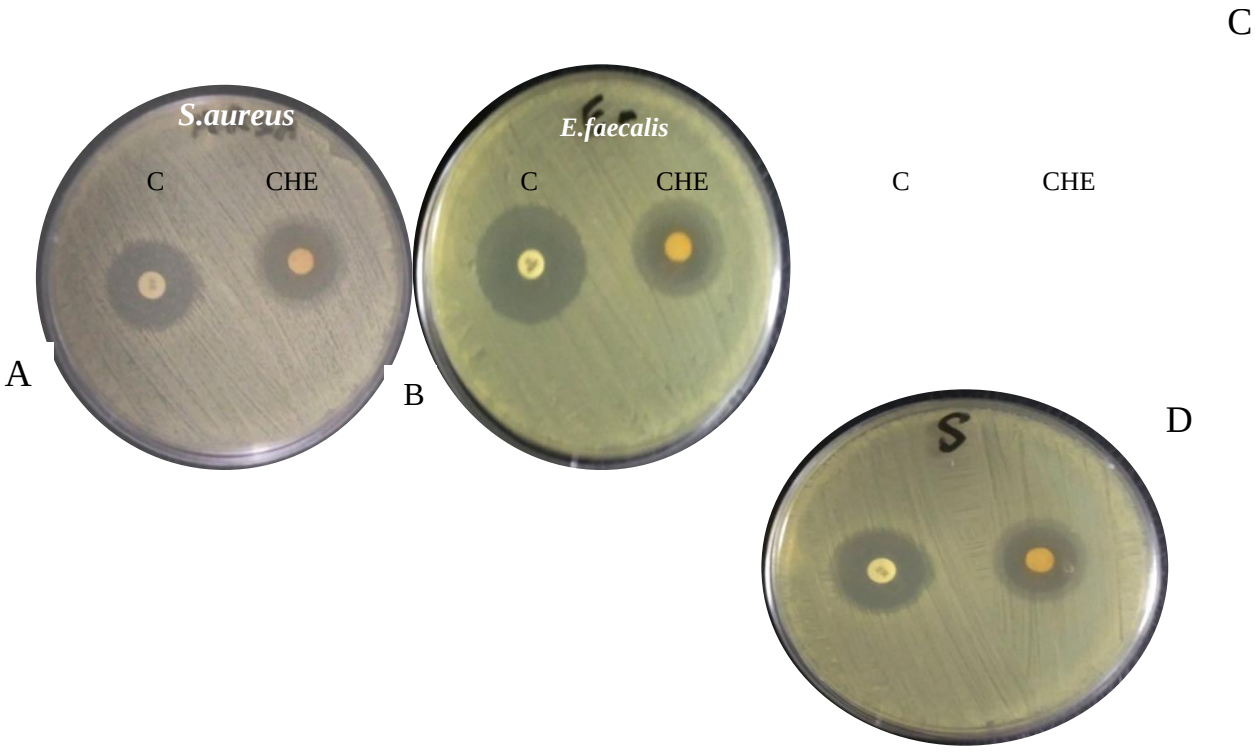

Fig. 1. Antibacterial activity of CBH ethanolic extract against +ve Gram bacteria: *S.aureus* (**A**); *E. faecalis* (**B**); *S.pyogenes* (**D**) using amikacin (**C**) as a positive control.

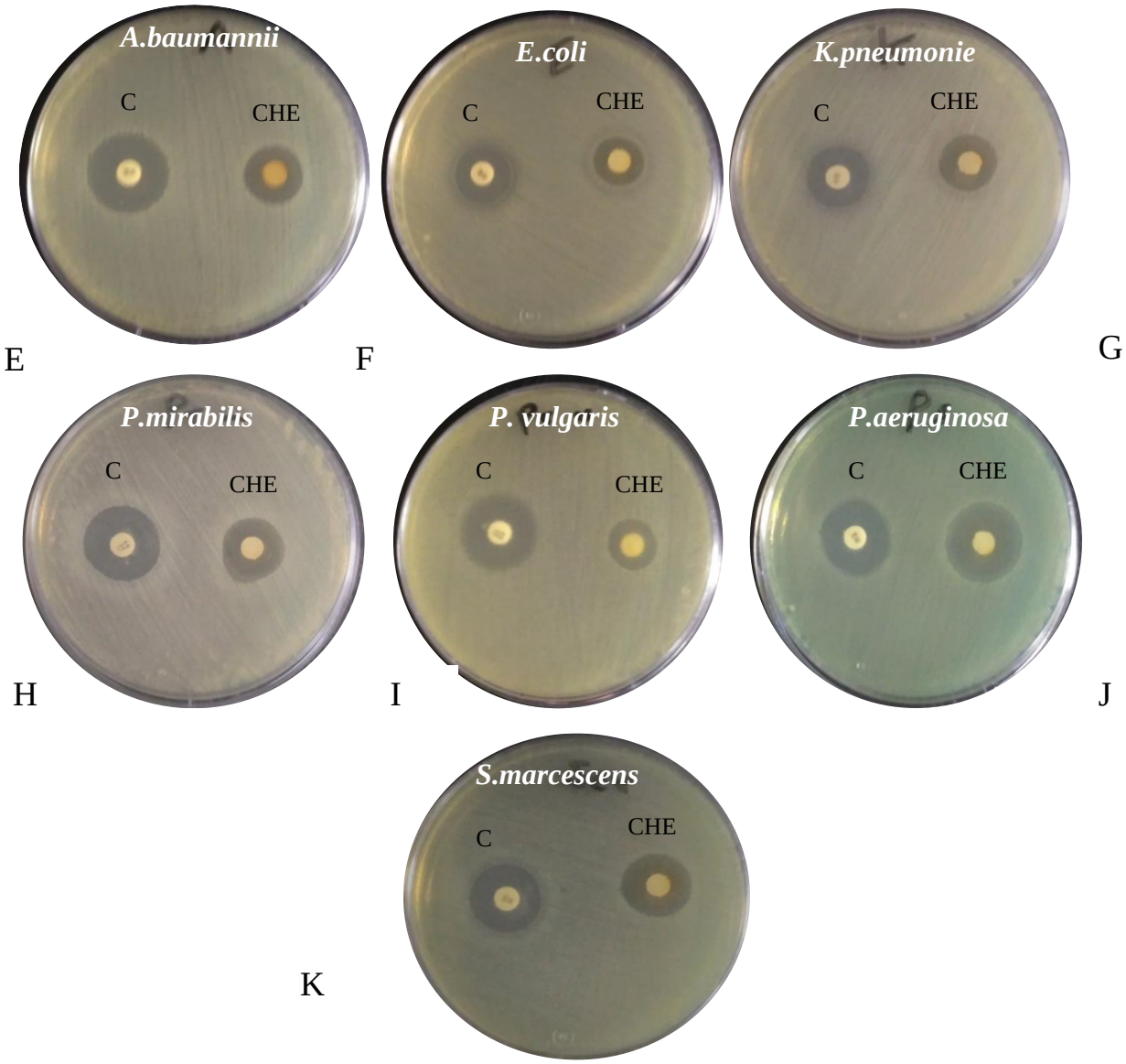

Fig. 2. Antibacterial activity of CBH ethanolic extract against -ve Gram bacteria: *A. baumannii* (**E**); *E. coli* (**F**); *K.pneumoniea* (**G**), *P.mirabilis* (**H**); *P. vulgaris* (**I**); *P.aeruginos* (**J**),*S. marcescens* (**K**) using amikacin (**C**) as a positive control.

4.3. Antifungal activity of Cocoa bean husk (CBH) ethanolic extract

Table (3) and Figure (3) show the antifungal activity of CBH extract. The growth of *A. niger* and *A. fumigatus* was completely inhibited by the ethanol extract of CBH, with inhibitory zones of 31.0 mm and 28.7 mm, respectively. With an average diameter of inhibition of 18.7 mm against *C. albicans*, the antifungal activity was much stronger than that of fluconazole (positive control), which was 15.0 mm. Davis and Stout [58] classified the potency of antifungal drugs according to the inhibition zone's diameter. Diameters of the inhibiting zone of < 5 mm, 5–10 mm, 10–20 mm, and ≥ 20 mm are classified as weak, medium, and strong, respectively. This result demonstrated that CBH had an antifungal effect that classified into the strong and extremely strong range. Secondary metabolite chemicals found in CBH have antifungal properties. According to Belwal *et al*. [59], phytochemical assays indicated the presence of flavonoids, alkaloids, and saponins in CBH extract with 70% ethanol, which has potential applications as antifungal agents. Alkaloids prevent fungal cells and other cells from synthesizing proteins, polysaccharides, DNA, and RNA [60]. Alkaloid chemicals genuinely function in the cell membrane by rupturing the chitin and glycan polymers, which results in cell death. Saponins can combine with sterols in a cell's membrane

to generate complexes that can result in membrane defects [61]. Additionally, flavonoids prevent the expansion of the fatty acid production pathway, which prevents the fungal cell walls formation [62].

Table 3: Antifungal activity of Cocoa bean husk (CBH) ethanolic extract against pathogenic fungal strains

Fungi	Zone of inhibition in mm (mean ± SD)	
	CBH ethanol extract	**Positive Control (Fluconazole)**
Aspergillus niger	31.0 ± 0.82	35.7 ± 0.47
Aspergillusfumigatus	28.7 ± 0.47	31.3 ± 0.94
Candida albicans	18.7 ± 0.47	15.0 ± 0.82

One-way ANOVA was used for statistical analysis, and significance ($P<0.05$) was assessed using Duncan's multiple comparison tests.

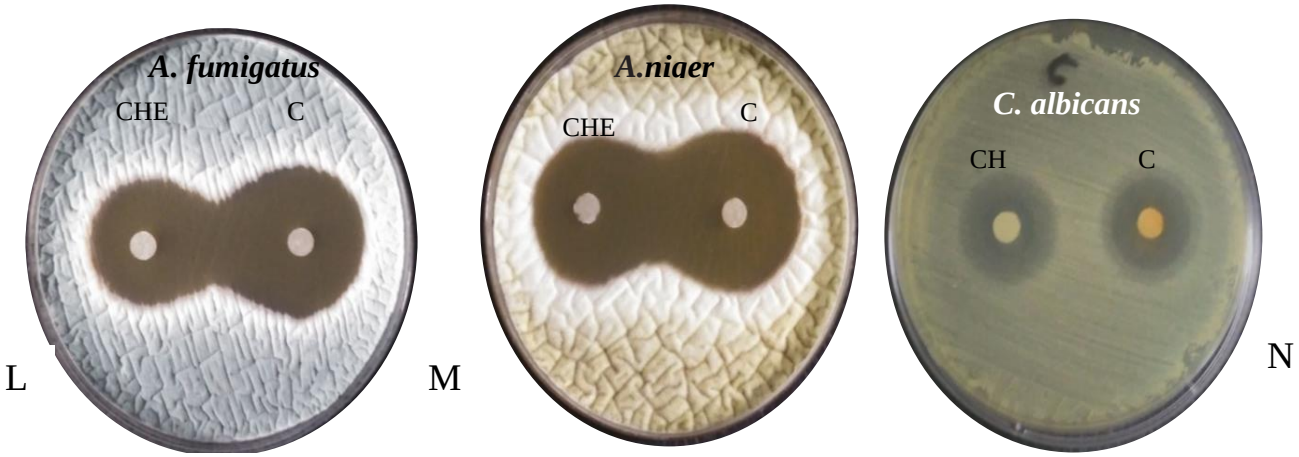

Fig. 3. Antifungal activity of CBH ethanolic extract against: *A.fumigatus* (**L**); *A.niger* (**M**); *C. albicans* (**N**), using fluconazole (**C**) as a positive control.

4.4. The minimum inhibitory concentration of Cocoa bean husk (CBH)

Table (4) presents the lowest inhibitory concentration (MIC) of CBH ethanol extracts on specific strains of bacteria and fungal strains. Depending on the tested microorganism, the values of MIC varied between 31.2 µg/ml and 250 µg/ml. Our results are lower than those found by [1,63], but they correspond with [64,65].

Table 4: Minimum inhibitory concentration values of Cocoa bean husk (CBH) ethanolic extract

	Microorganism	**MIC µg/ml**
Bacterial strains	*S.aureus*	31.2
	Enterococcus faecalis	125
	Streptococcus pyogenes	250
	Acinetobacter baumannii	62.5
	Escherichia coli	125
	Klebsiella pneumoniea	125
	Proteus mirabilis	62.5
	Proteus vulgaris	125
	Pseudomonas aeruginosa	31.2
	Serratia marcescens	62.5
Fungal strains	*Aspergillus niger*	62.5
	Aspergillus fumigatus	31.2
	Candida albicans	250

4.5. Antioxidant Effects of Cocoa bean husk (CBH) extract in vitro

The antioxidant activity test of the CBH extract was performed using the DPPH radical scavenging method. The concentrations that were examined were 100, 50, 25, 12.5, 6.25, 3.12, and 1.56 g/mL. Table (5) demonstrated that the antioxidant properties of CBH extract were equivalent to that of standard ascorbic acid. The percentage of antioxidant action expanded for each tested dose in a dose-dependent manner. According to [66], the antioxidant activities of CBH extract show health benefits in this regard. DPPH free radicals could be reduced by it (IC50= 264.8675). Studies carried out by [67] revealed a beneficial connection between phenolic content and antioxidant activity. Additionally, the antioxidant activity and total phenolic content of cocoa bean husk (CBH) were measured by [68]. According to the findings, the total phenolic content of the 70% ethanol extract and the acetone extract were 49.92 GAE/g and 94.92 GAE/g, respectively. The acetone extract had 44.11% and the 70% ethanol extract had 88.16% of its antioxidant activity, respectively.

Table 5: Free radical scavenging activity of Cocoa bean husk (CBH) extract against Positive Control ascorbic acid (Standard) ascorbic acid. (Mean ± SE)

(Conc. µg/mL)	DPPH scavenging % of Cocoa bean husk (CBH)extract	DPPH scavenging %of Positive Control ascorbic acid (Standard)
100	97.7 ± 0.05 [a]	90.4±0.005[b]
50	94.5± 0.03 [a]	85.8±0.01[b]
25	91.2± 0.02 [a]	82.2±0.02[b]
12.5	84.2±0.01[a]	70.1±0.04[b]
6.25	78.6±0.01[a]	63.5±0.03[b]
3.12	66.2±0.01[a]	55.4±0.03[b]
1.56	56.6 [a]±0.01[a]	42.5±0.03[b]

One-way ANOVA was used for statistical analysis, and significance (P<0.05) was assessed using Duncan's multiple comparison tests.

4.6. In vitro anti-inflammatory potency

Table (6) showed how the CBH extract's anti-inflammatory properties were determined in vitro using the human red blood cell membrane stabilization technique. The findings we obtained showed that the high anti-inflammatory activity of CBH extract was similar to that of standard (indomethacin). When compared to the hemolytic percents of CBH extract, which were 98.3 ± 0.01, 94.6 ± 0.03, 90.2 ± 0.02, 88.3 ± 0.01, 76.4 ± 0.05, and 65.1 ± 0.03, respectively, indomethacin generated an inhibition of 95.2 ± 0.01, 90.5 ± 0.02, 83.2 ± 0.01, 74.1 ± 0.02, 61.3, and 52.3± 0.01, respectively. In this regard, [69] investigation of cacao extract anti-inflammatory characteristics (Lipopolysaccharide-induced RAW264.7 cells were used) revealed that it could suppress the production of nitric oxide and prostaglandin E2 by down-regulating the cyclooxygenase-2 and inducible nitric oxide synthesis expression levels. Furthermore, by preventing the nuclear factor-kappa B signaling pathway, phosphatidylinositol-3-kinase/protein kinase B, and mitogen-activated protein kinases from being activated., cocoa extract showed increased anti-inflammatory activity.

Table 6: Effect of standard indomethacin and cocoa bean husk (CBH) extract on % of hemolysis prevention, (Mean ± SE)

Concentration. ug/ml	% of haemolysis prevention Positive Standard (Indomethacin)	% of haemolysis prevention (CBH) extract
1000	95.2±0.01	98.3±0.01
800	90.5±0.02	94.6±0.03
600	83.2±0.01	90.2±0.02
400	74.1±0.02	88.3±0.01
200	61.3 ±0.01	76.4±0.05
100	52.3 ±0.01	65.1±0.03

One-way ANOVA was used for statistical analysis, and significance (P<0.05) was assessed using Duncan's multiple comparison tests.

4.7. Cytotoxicity effect of CBH extract on the mnormal wi38cells

Figures (4, 5) demonstrated that the CBH extract's cytotoxicity was determined by the assay of MTT on WI-38 cell, a diploid human normal cell line. Based on the MTT procedure, the values of IC50 for the CBH extract were 302.96 and 382.99 ug/mL. According to [70], the death rate of brine shrimp was less than 50% at any concentration after 24 hours, indicating that cocoa bean husk (CBH) extract was non-toxic in this regard. These outcomes correspond with the conclusions of [71], who found that even at high concentrations, T. cacao may not cause much harm to leukocyte DNA. According to [72], there was no substantial DNA damage caused by cocoa bean husk (CBH) extracts on mammalian cells at doses of up to 10 µg/ml. S Furthermore, [73] observed that at concentrations of 100 pg/ml, 50 pg/ml, 25 pg/ml, 12.5 pg/ml, 6.25 pg/ml, 3.125 pg/ml, and 1.56 pg/ml, cocoa husk extract did not exhibit any harmful effects on the vitality of the periodontal ligament fibroblasts' basic cells.

control
WI-38cells

Organism :	*Homo sapiens*, human
Tissue :	lung
Cell Type :	fibroblast
Culture Properties :	adherent
Disease :	normal

Fig. 4. Control of WI-38 normal cells

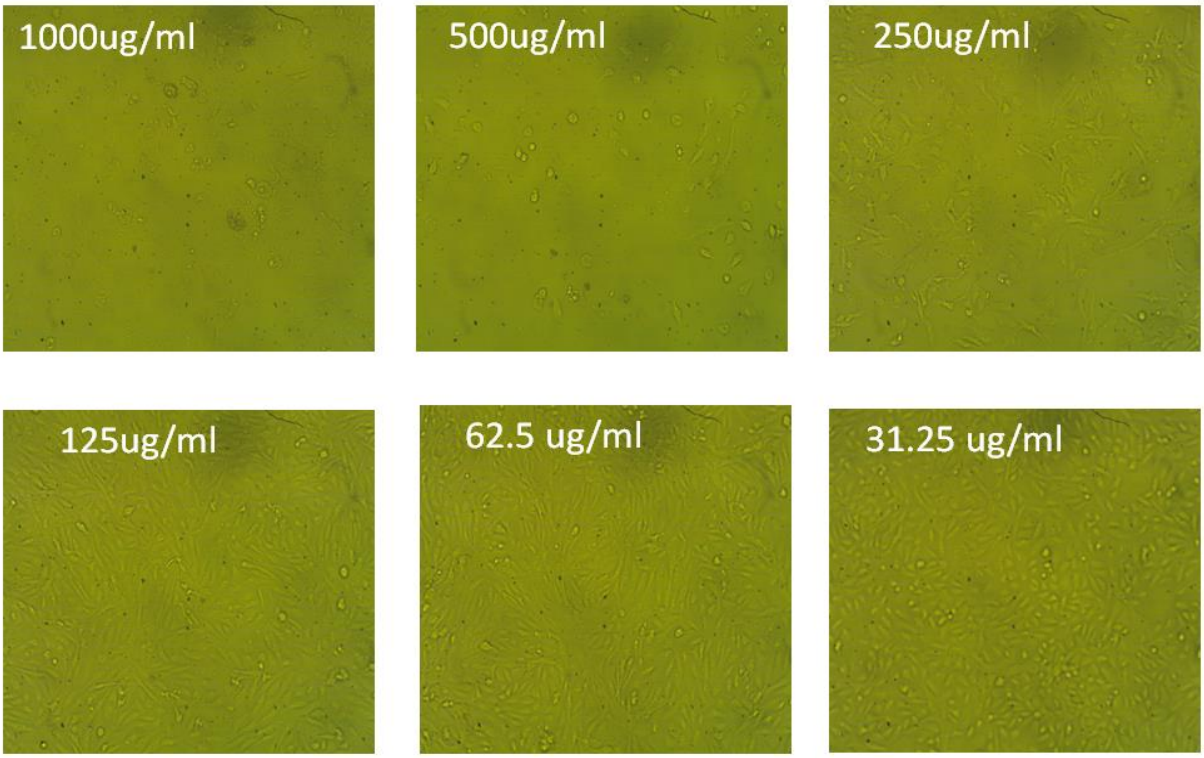

Fig 5. Extensive morphological alterations in the cytoskeleton or on the surface of cells were seen and linked with cell survival at different doses in comparison to control at varying concentrations of cocoa bean husk (CBH) extract, 1000, 500, 250, 125, 62.5 and 31.25ug/mL

4.8. HPLC analysis of main phenolic compounds in CBH extract:

The phenolic compounds are those with an aromatic ring that include their functional derivatives and one or more hydroxy substituents [74]. Table (7) presents the main phenolic compound concentrations detected by HPLC in the current investigation. The compounds that were detected included (+)-catechin, Chlorogenic acid, Ellagic acid, Syringic acid, Gallic acid, Quercetin, Naringenin, Daidzein, Coumaric acid, Rutin, Rosmarinic acid, Hesperetin, and Vanillin. As shown in Figure (6), (+)-catechin is the most prevalent (13.36 mg/g). Chlorogenic acid was the main phenolic acid recovered, with concentration (2.37 mg/g). However, the phenolic compounds with lower concentrations were rosmarinic and ferulic acids. Our findings match with those of Jokić *et al.* [75], who found that the primary phenolic component in CBH extract was catechin. Catechin is a type of flavonoid found in cocoa beans and shells, and it has been associated with several of health benefits. Additionally, some studies have suggested that (+)-catechin can be used to prevent the growth of pathogenic microorganisms [76]. According to the theory of membrane disruption, catechins cause lateral expansion and membrane disruption when they intercalate into the lipid bilayer. Additionally, catechins oxidize in the cell culture medium and produce hydrogen peroxide, which damages DNA and causes protein oxidation [77]. Also, catechin has anti-inflammatory effects by controlling inflammatory cytokines, lowering the generation of reactive oxygen species, and promoting neutrophil migration [78]. Polyphenols are the most widely distributed phyto-antioxidants Phenol groups regulate protein phosphorylation by preventing lipid peroxidation [79]. However, scavenging reactive oxygen species (ROS) is the direct mechanism, while the suppression of pro-enzyme that contribute to oxidant stress and the rise of antioxidant enzymes constitute the indirect mechanism [80]. According to Yahya *et al*. [81], cocoa husk is regarded as a waste product that is high in polyphenols. These beneficial components may be responsible for the pharmacological qualities of cocoa shells. According to several studies, phenolic compounds have anti-inflammatory, antibacterial, antifungal, and antioxidant properties [82, 83, 84].

Table 7: Contents of phenolic compounds determined by HPLC

Peak	Compounds	RT	Conc. (mg/g)
1	**Gallic acid**	3.574	0.323
2	**Chlorogenic acid**	4.189	2.37
3	**Catechin**	4.384	13.363
4	**Syringic acid**	6.220	0.40
5	**Pyro catechol**	6.689	0.004
6	**Rutin**	6.948	0.023
7	**Ellagic acid**	7.247	0.679
8	**Coumaric acid**	8.385	0.069
9	**Vanillin**	9.183	0.012
10	**Ferulic acid**	9.759	0.001
11	**Naringenin**	10.156	0.091
12	**Rosmarinic acid**	11.782	0.021
13	**Daidzein**	15.923	0.083
14	**Querectin**	17.074	0.296
15	**Cinnamic acid**	19.246	0.002
16	**Kaempferol**	20.571	0.001
17	**Hesperetin**	21.212	0.014

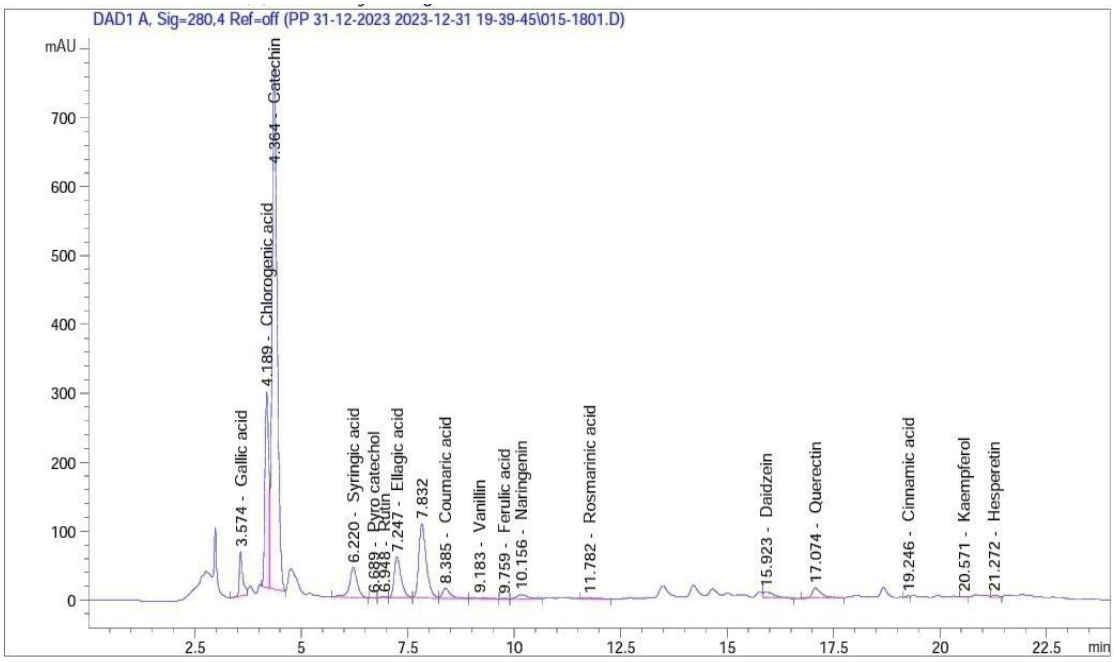

Fig. 6. Profile of total Phenolic components of cocoa analyzed by HPLC

Conclusion

One excellent source of naturally occurring bioactive compounds is plants that have shown promise in the medical and pharmaceutical fields. According to the study's findings, all test pathogenic bacteria, including Gram-negative and Gram-positive bacteria may be inhibited by ethanol extract of cocoa bean husk (CBH). Additionally, CBH extract tends to prevent tested human pathogenic fungi. The extract has no cytotoxic effect on normal cells. The extract exhibited strong anti-

inflammatory and antioxidant properties. The presence of polyphenolic chemicals in the ethanolic extract of cocoa bean husk (CBH) may be the cause of the extracts' biological activity. Because of this, there's a good potential that, with additional purification and appropriate processing, they can be used as antimicrobial drugs.

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