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Antimicrobial, Antioxidant and Anti-inflammatory Characteristics of Combination (*Cassia fistula* and *Ocimum basilicum*) Extract as Natural Preservative to Control & Prevent Food Contamination

Gamal M. Hamad[1,3,*], Amira M.G. Darwish[1,3], Marwa M Abu-Serie[2,3], Sobhy A. El Sohaimy[1,3]

[1]Food Technology Department, Arid Lands Cultivation Research Institute (ALCRI)
[2]Medical Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI)
[3]City of Scientific Research and Technology Applications (SRTA-CITY), New Borg El-Arab City, Alexandria, Egypt
*Corresponding author: hamad6751@yahoo.com

Abstract **Objectives:** *Cassia fistula* and *Ocimum basilicum* which are assumed to contain the active components and which are renewable source in fighting infections of microorganisms. The aim of this study was to investigate the antimicrobial activity, the antioxidant activity and anti-inflammatory properties of *Cassia fistula* and *Ocimum basilicum* and their mix. **Methods and Materials:** *Cassia fistula* and *Ocimum basilicum* exhibited antimicrobial activity against all of the tested bacteria and yeasts except for *Asperigillus spp*. that showed high resistance. **Results and Discussion:** The minimum inhibitory concentration (MIC) values for bacteria and yeast ranged from 3.1 to 6.2 mg/ mL. The antioxidant activity of the *Cassia fistula* and *Ocimum basilicum* was evaluated by using DPPH radical scavenging assay. In addition, the amount of total phenolic content (TPC) of *Cassia fistula*, *Ocimum basilicum* and their mix was 64.71, 96.42 and 113.07 mg/g, while total flavonoids content was 16.33, 32.66 and 79 mg/g respectively. Vanillin, quercetin and cinnamic acid marked the phenolic profile of the mix. Cytotoxicity results indicated high safety use of the two plants. **Conclusion:** Our data confirmed convenient and safe use of *C. fistula*, *O. basilicum* and their mix as natural antimicrobial and antioxidant. The mix of both extracts can be recommended as natural preservative to be used in food processing to control and prevent food contamination.

Keywords: *Cassia fistula and Ocimumbasilicum, Antimicrobial activity, antioxidant, anti-inflammatory, cytotoxicity assay, foodborne pathogens*

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1. Introduction

Food poisoning in developing countries mostly resulted from bacteria such as; *Escherichia coli, Staphylococcus aureus*, *Bacillus cereus* and *Salmonella spp.* [1,2,3,4,5] Most common materials used for controlling food spoilage are chemicals that have serious effects on human health. Consequently, great efforts have been spent for discovering safe effective natural materials which could be used in food preservation [6,7,8].

History supports using of medicinal plants, due to presence of vast and diverse assortment of organic compounds that can produce a definite physiological action on the human body [9] The antimicrobial activity exhibited by plant extracts against food poisoning bacteria has been demonstrated by several researchers. [10,11,12] Most important of such compounds are alkaloids, tannins, flavonoids, terpenoids, saponins and phenolic compounds. A great interest in these compounds is relied to their therapeutic performance and low toxicity. [13] A number of such compounds have been isolated from plants which could be used to inhibit the growth of bacterial and fungal pathogens and to quench ROS with possibly novel mechanisms of action to the host cell. [14]

Cana fistula (*Cassia fistula, Leguminosae*), is an ornamental tree distributed in different parts of the world including Asia, South Africa, Mexico, China, East Africa and Brazil. Extract of this plant is been heavily used as broad-spectrum antimicrobial agent. [15] Basil (*Ocimum basilicum*, *Lamiaceae*), is one of the oldest herbs/spices that is known for its ornamental and therapeutic importance. It is originated from India, and has been reported to be hepatoprotective, immunomodulatory, antihyperglycemic, hypolipidemic, antitoxic, anti-inflammatory, antibacterial, antifungal and chemopreventive agent [16,17,18].

The main aim of the present research was to focus on phytochemical content of two medicinal plant extracts; cana fistula (*Cassia fistula*), basil (*Ocimum basilicum*)

and their mix, examine their antimicrobial potentials against biological food contaminants either bacteria or fungi and investigation of their safety and cytotoxicity considerations to suit food applications.

2. Materials and Methods

2.1. Plant Material Samples and Preparation

Cana fistula (*Cassia fistula*) and basil (*Ocimum basilicum*) leaves was obtained from Experimental Farm of City of Scientific Research and Technological Applications, Alexandria, Egypt. The leaves plant samples were dried at (40°C/ 24hr). Grounded leaves were extracted in deionized water (1:20 w/v), centrifuged at 3000 rpm for 15 min and filtrated. Aqueous plant extract was lyophilized using vacuum freeze-dryer (Model FDF 0350, Korea) to obtain the dried powder that freeze stored for analysis. Table 1 contains description of the two studied plants.

Table 1. Description of studied plants

Parameter	Canafistula	Basil
Botanical name	(*Cassia fistula*)	(*Ocimum basilicum*)
Family	*Fabaceae/ Leguminosae*	*Lamiaceae*
English name	Cana fistula, golden rain tree	Basil, Saint-Joseph's-wort
Vernacular name (in Egypt)	khiārshambar	Rehan
Part used	Leaves	Leaves
Checked names on Plant list http://www.theplantlist.org;	http://www.theplantlist.org/tpl1.1/search?q=Cassia+fistula	http://www.theplantlist.org/tpl1.1/search?q=Ocimum+basilicum

2.2. Antimicrobial Activity

2.2.1. Microorganisms and Culture Conditions

Pathogenic bacteria strains used were; five Gram-positive strains; *Bacillus cereus* EMCC 1006, *Staphylococcus aureus* EMCC 1351, *Streptococcus pyogenes* EMCC 1772, *Streptococcus mutans* EMCC 1815 and *Clostridium perfringens* EMCC1574 and three Gram-negative strains; *Salmonella spp., Escherichia* coli ATCC 25922 and *Klebsiella pneumoniae* EMCC 1637. *Tested* yeast strains were; *Saccharomyces cerevisiae* DSM 70487, *Pichia memberanifaciens* CBS 107*, Aureobasidium pullulans* ATCC 42023, *Schizosaccharomyces octosporus* EMCC 93, *Rhodotorula glutinis* EMCC 175, *Kluyveromyces Lactis* DSM 70800*, Hansenula anomala* CBS 5759 and two Candida spp.; *Candida utilis* EMCC 41, *Candida albicans* ATCCMYA-2876. Fungi strains were; *Aspergillus niger* EMCC 72, *Aspergillus flavus* EMCC 274 and *Aspergillus parasiticus* EMCC 886^T. All strains were obtained from Microbiological Resources Center (MERCIN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. The strains were maintained by; the Department of Food Technology, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications, Egypt in 60% glycerol/ LB culture at -80°C.

2.2.2. Minimum Inhibitory Concentration (MIC) Determination

The bacterial strains were grown in nutrient broth at 37°C, whereas the fungi and yeast strain was grown in Sabouraud dextrose at 28°C for 24h. A set of 6 concentration of reconstituted plant water extracts (75, 50, 25, 12.5, 6.25 and 3.1 mg/ mL), were examined to determine the minimum inhibitory concentration (MIC) of each against a specific pathogenic strain. [19,20] The zone of inhibition was calculated by measuring the diameter of the inhibition zone around the well (mm), including the well diameter. The readings were taken in three different fixed directions in all triplicates and the average values were tabulated.

2.2.3. Phytochemical qualitative analysis

The qualitative phytochemical analysis was performed Test for tannins, flavonoids, alkaloids, reducing sugars, volatile oils, glycosides, amino acids and proteins, saponins (Foam Test), terpenoids (Salkowki's Test), Steroids according to. [21,22]

2.2.4. Total Phenolic Content Determination

The total phenolic content of *Cassia fistula* and *Ocimum basilicum* leaves aqueous extracts was determined by Folin-Ciocalteu spectrophotometric method. [20] Aliquate of 0.1 mL of Folin-Ciocalteu reagent was added to 2 mL of reconstituted extract. The mixture was allowed to stand for 15 min. Then, 3 mL of saturated sodium carbonate 2% (Na_2CO_3) was added. The mixture was allowed to stand for 30 min at room temperature and the total phenolic content was determined using spectrophotometer (Labo America, USA) at 760 nm. Gallic acid was used as a standard. Total phenol values are expressed in terms of mg of gallic acid equivalent per gram of the sample using the linear regression equation obtained from the standard gallic acid calibration curve y = 0.014x - 0.168. All samples were analyzed in triplicates.

2.2.5. HPLC Conditions for Phenolic Compounds Quantification

Quantification of phenolic compounds of *Cassia fistula* and *Ocimum basilicum* leaves water extract was determined via High Performance Liquid Chromatography (HPLC) according to. [23] Ten phenolic standards of phenolic compounds were used; gallic acid, caffeic acid, coumaric, syringic acid, vanillin, cinnamic acid and pyrogallol, catechin, quercetin and rutin. Agilent 1260 infinity HPLC series (Agilent, USA), equipped with quaternary pump, a Zorbax Eclipse plus C18 column 100 mm x4.6 mm i.d., (Agilent technologies, USA) operated at 25°c was used for phenolic compound analysis. The injected volume was 20µ: VWD detector set at 284 nm. The separation is achieved using a ternary linear elution gradient with (A) HPLC grade 0.2% H_3PO_4 (v/v), (B) methanol and (c) acetonitrile. The injection volume for *Cassia fistula* and *Ocimum basilicum* leaves aqueous extracts was 1g/10 mL. All standards were dissolved in ethanol and injected with the following concentrations; gallic = 12 µg/mL, caffeic acid = 12 µg/mL, coumaric acid = 8 µg /mL; syringic acid = 8 µg/mL, vanillin = 8 µg/mL, cinnamic acid = 4 µg/mL, pyrogallol = 65 µg/mL,

catechin = 40 µg/mL, quercetin = 32 µg/mL and rutin = 40 µg/ mL. Compounds were identified by comparing their retention times and UV–Vis spectra with those of the standards, while their concentrations were calculated depending on the area under the peak of standards.

2.2.6. Antioxidant Activity Evaluation

The ability of the plant extracts to scavenge DPPH free radicals was assessed by the standard method of [20,24]. Adopted with suitable modifications [22]. A stock solution of each extract was prepared in methanol to final concentration 1 mg/mL. Plant serial dilutions were made; about 1mL of each dilution was mixed with 1mL of methanolic solution of DPPH in a concentration of 1 mg/mL. After 30 min incubation in darkness, the absorbance was measured at 517 nm. IC50 values were estimated from the % inhibition versus concentration plot, using a non-linear regression algorithm, after inhibition percentage was calculated using following equation;

$$\text{Inhibition \%} = \left[\left(\text{A of control} - \text{A of sample}\right) / \text{A of control}\right] \text{x} 100.$$

2.2.7. Cytotoxicity Safe Dose Determination Assay

In 96-well plate, 1 x 10^5 WBCs / well were mixed with 100 µL of serial dilutions of extracts and standard anti-inflammatory drug (hydrocortison). The plate was incubated in humidified atmosphere at 37°C, 5% CO_2 and 90% relative humidity for 72h. At the end of the incubation period; 20 µL of MTT (5 mg/mL in PBS) was added per well and incubated in CO_2 incubator for 3h. Later, plates were centrifuged at 2000 rpm for 10 min to discard MTT solution and then 100 µL of DMSO was added and the absorbance was read at 570 nm using ELISA reader (BMG Lab Tech, Germany) to estimate the percentage of cell viability. The safe dose (EC_{100}, 100% cell viability) was calculated from the relation between the cell viability and different extracts or dexamethasone using GraphpadInstat software.

2.2.8. Anti-inflammatory Assay for Effective Concentration Assessment

In each well of 96-well plate, 1 x 10^5WBCs were seeded and mixed with 1 mg/ml LPS for induction of inflammation. After 24 h in CO_2 incubator, cells treated with 100 µL of medium, extracts or standard hydrocortison at serial concentrations of the safe dose For 72 h. Then MTT solution was added to each well and the same procedure as described above. The stimulation index (SI) that defines as the ratio of absorbance of extract- or hydrocortison-treated LPS-stimulated WBCs versus the absorbance of untreated WBCs was calculated for determination of the effective concentration (EC) of each extract and hydrocortison. The EC is the concentration which is able to reverse the abnormal SI value of LPS-stimulated cells to the normal value (SI ~ 1).

2.2.9. TNF-α and NO Assays

Supernatants of LPS-stimulated WBCs and extracts or hydrocortisone-treated WBCs at their EC were collected, after centrifugation, for TNF-α and NO quantification. TNF-α was estimated using ELISA kit following the manufacture´s protocol (Ray Biotec, USA). NO level was assessed based on the Griess reaction [25], 50 µl of supernatants were mixed with 100 µl of Griess reagent in 96-well microtiter plate and incubated for 5 min at 25°C. Then, the absorbance of the purple-azo-dye product was detected at 490 nm using an ELISA reader. NO concentration was determined by comparison with a standard curve of sodium nitrite.

2.2.10. Real Time Quantitative Polymerase Chain Reaction (qRT-PCR) of COX-2

Total RNA was extracted from untreated cells, LPS-stimulated cells and extract and hydrocortisone-treated LPS-stimulated cells using RNA Purification Kit following the manual protocol (Thermo Fisher Scientific, USA). Then cDNA was generated by reverse transcriptase-PCR using the cDNA synthesis kit (Thermo Fisher Scientific, USA). Levels of gene expression of cyclooxygenase (COX-2) as target genes and β-actin (reference gene) were quantified by RT-PCR (Qiagen, Germany). The gene-specific forward and reverse primers are 5′-ATCATTCACCAGGCAAATTGC-3′ and 5′-GGCTTCAGCATAAAGCGTTTG-3′, respectively. The reaction mixture contained 12.5 µL of 2X SYBR green master mix, 0.3 µL of 10 µM forward primer, 0.3 µL of 10 µM reverse primer, cDNA template and nuclease-free water. The quantitative PCR program was applied as 95°C for 15 min followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 1 min and extension at 72°C for 30 sec. The change in expression of target genes was calculated by the $2^{-\Delta\Delta CT}$ method.

2.3. Statistical Analysis

Statistical analysis was performed using Analytical Software SPSS® 13.0 (Statistical Package for the Social Sciences) (2005).

3. Results and Discussion

3.1. Antimicrobial Potentials

Antimicrobial activity of each extract was studied individually prior to investigating antimicrobial potentials of their mix. Antimicrobial activity of cana fistula aqueous extract against tested pathogenic bacteria was exhibited in (Table 2), expressed by inhibition zone diameters and MICs (Minimum Inhibition Concentration). Results showed good antibacterial activity against, Gram-positive strains, *Staphylococcus aureus* EMCC1351, *Streptococcus mutans* EMCC1815 with MIC of 12.5 mg/mL, *Bacillus cereus* EMCC1006 and *Clostridium perfringens* EMCC1574 with MIC 6.2 mg/mL, while in Gram-negative strains the extract only affected *Escherichia coli* ATCC25922 with MIC of 12.5 mg/mL. C. fistula aqueous extract did not show antimicrobial effect on *Streptococcus pyogenes* EMCC1772, *Salmonella spp. or Klebseilla pneumonia* ATCC12296. These results are consistent with previous reports regarding “Gram-positive” and “Gram-negative” bacteria, which reported that the later are more resistant. Antimicrobial activity of Cassia fistula supports folkloric use in the treatment of some diseases as broad-spectrum antimicrobial agents. [26]

Table 2. Inhibition zone diameters and MICs of cana fistula (*Cassia fistula*) aqueous extract against bacterial strains

Pathogenic strain	Inhibition zone diameter (mm)**						
	75*	50*	25*	12.5*	6.2*	3.1*	MIC
Gram-positive bacteria							
Bacillus cereus EMCC1006	25±1.58	20±1.00	18±1.44	9±2.15	5±1.12	ND	6.2
Staphylococcus aureus EMCC1351	20±2.43	15±2.00	11±2.36	6±2.00	ND	ND	12.5
Streptococcus pyogenes EMCC1772	ND	ND	ND	ND	ND	ND	ND
Streptococcus mutans EMCC1815	21±2.64	19±2.41	12±2.33	5±2.00	ND	ND	12.5
Clostridium perfringens EMCC1574	24±1.98	20±1.67	18±2.34	9±2.40	5±1.00	ND	6.2
Gram-negative bacteria							
Salmonella spp.	ND	ND	ND	ND	ND	ND	ND
Escherichia coli ATCC25922	20±3.12	16±1.62	7±1.68	5±1.94	ND	ND	12.5
Klebseilla pneumonia ATCC12296	ND	ND	ND	ND	ND	ND	ND

Data represented are average of triplicates
MIC; Minimum Inhibition Concentration
*Concentrations of extract and MIC are in mg/mL
**Diameter included 5 mm well diameter
ND; Not detected.

Table 3. Inhibition zone diameters and MICs of cana fistula (*Cassia fistula*) aqueous extracts against yeast and fungi strains

Pathogenic strain	Inhibition zone diameter (mm)**						
	75*	50*	25*	12.5*	6.2*	3.1*	MIC
Yeast							
Saccharomyces cerevisiae DSM70487	20±2.17	19±2.31	16±1.99	13±2.17	7±1.88	ND	6.2
Pichia memberanifaciens CBS107	23±3.24	18±3.26	14±3.72	8±1.87	ND	ND	12.5
Aureobasidium pullulans ATCC42023	21±2.51	17±1.67	12±3.41	8±2.85	ND	ND	12.5
Schizosaccharomyces octosporus EMCC93	22±2.73	19±1.65	13±1.84	9±2.59	ND	ND	12.5
Rhodo torulaglutinis EMCC175	20±2.81	17±2.46	13±1.64	8±1.58	ND	ND	12.5
Kluyveromyces Lactis DSM70800	23±2.15	18±1.75	15±1.68	11±1.36	5±1.47	ND	6.2
Hansenula anomala CBS5759	24±2.35	18±2.73	14±2.39	10±2.14	6±2.43	ND	6.2
Candida utilis EMCC41	22±2.16	19±2.03	15±1.69	9±2.51	ND	ND	12.5
Candida albicans EMCC105	21±1.68	16±2.57	11±2.43	5±2.16	ND	ND	12.5
Fungi							
Aspergillus niger EMCC 72	ND	ND	ND	ND	ND	ND	ND
Aspergillus lavus EMCC 274	ND	ND	ND	ND	ND	ND	ND
Aspergillus parasiticus EMCC 886^{T}	ND	ND	ND	ND	ND	ND	ND

Data represented are average of triplicates
MIC; Minimum Inhibition Concentration
*Concentrations of extract and MIC are in mg/mL
**Diameter included 5 mm well diameter
ND; Not detected.

Table 4. Inhibition zone diameters and MICs of basil (*Ocimum basilicum*) aqueous extract against bacterial strains

Pathogenic strain	Inhibition zone diameter (mm)**						
	75*	50*	25*	12.5*	6.2*	3.1*	MIC
Gram-positive bacteria							
Bacillus cereus EMCC 1006	22±1.54	17±2.34	6±1.35	ND	ND	ND	25
Staphylococcus aureus EMCC1351	23±2.17	19±1.68	8±1.49	5±1.03	ND	ND	12.5
Streptococcus pyogenes EMCC1772	21±2.45	8±2.36	5±0.88	ND	ND	ND	25
Streptococcus mutans EMCC1815	21±2.35	17±3.20	10±2.44	5±0.04	ND	ND	12.5
Clostridium perfringens EMCC1574	20±2.74	15±3.21	5±.33	ND	ND	ND	25
Gram-negative bacteria							
Salmonella spp.	20±3.67	12.95±1.81	6±0.44	ND	ND	ND	25
Escherichia coli ATCC25922	21±3.19	19±2.58	9±0.37	ND	ND	ND	25
Klebseilla pneumonia ATCC12296	20±1.64	11±3.11	5±0.33	ND	ND	ND	25

Data represented are average of triplicates
MIC; Minimum Inhibition Concentration
*Concentrations of extract and MIC are in mg/mL
**Diameter included 5 mm well diameter
ND; Not detected.

Table 3 fulfills the antimicrobial profile of C. fistula by illustrating inhibition zone diameters and MICs of cana fistula aqueous extracts against yeast and fungi strains. Obtained results indicated remarkable antifungal impact against yeasts where, wide inhibition zones against yeast strains were recorded by C. fistula using concentration of 75 mg/mL that varied between (20 – 24 mm) with low effective MICs (6.2 and 12.5 mg/mL) against the nine

yeast strains. Unfortunately used *Aspergillus* spp. were more resistant to be affected by cana fistula aqueous extracts giving negative results. The antifungal effect of *C. fistula* was earlier confirmed by [27], who claimed that substances contained in the leaf extracts may have similar mechanism that could be related to fluid leaks in cells.

Table 4 represented inhibition zone diameters and MICs of basil aqueous extract against bacterial strains. Good antibacterial effect was obtained by basil against Gram-positive strains *Staphylococcus aureus* EMCC1351 and *Streptococcus mutans* EMCC1815 with MIC of 12.5 mg/mL. On the other hand, average antibacterial impact with MIC of higher concentration (25 mg/mL) was recorded against, Gram-positive strains, *Bacillus cereus* EMCC 1006, *Streptococcus pyogenes* EMCC1772 and *Clostridium perfringens* EMCC1574 and against all used Gram-negative strains, *Salmonella spp., Escherichia coli* ATCC 25922 and *Klebseilla pneumonia* ATCC 12296. Marwat et al. (2011) [28] reported antibacterial activities of O. basilicum against bacterial genera; *Bacillus, Escherichia coli* and *Staphylococcus aureus.*

Table 5 exhibited inhibition zone diameter and MICs of *O. basilicum* aqueous extracts against yeast and fungi strains. The most affected yeast strains amongst the nine used strains were *Kluyveromyces Lactis*DSM70800 and *Hansenulaanomala*CBS5759 with low MIC (6.2 mg/mL), while the less affected strain was *Candida albicans* EMCC105 that needed at least a MIC of (25 mg/mL) to be inhibited. The rest of yeast strains showed average MIC of (12.5 mg/mL). These results agrees with [29] who reported that *O. basilicum* showed best MIC against *C. albicans*. *Aspergillus* spp. did not show to be affected by basil aqueous extract as well. *O. basilicum* was reported to be a source of aroma compounds and essential oils containing biologically active constituents that possess insecticidal, fungistatic and antimicrobial properties. [30]

Antimicrobial activity of the two plant extracts mix against pathogenic bacteria was illustrated in (Table 6), expressed by inhibition zone diameter and MICs. Comparing with individual extracts antibacterial results (Tables 2 and 5), synergetic effect between the two plant extracts translated in elevation in inhibition zone diameters which ranged between (25 – 33 mm) at concentration of 75 mg/ mL and lowering MICs that recorded 6.2 mg/mL against all the nine bacterial strains. Synergism between plant extracts against pathogenic bacterial strains was previously reported by. [31]

Table 5. Inhibition zone diameters and MICs of basil (*Ocimum basilicum*) aqueous extracts against yeast and fungi strains

Pathogenic strain	Inhibition zone diameter (mm)**						
	75*	50*	25*	12.5*	6.2*	3.1*	MIC
Yeast							
Saccharomyces cerevisiae DSM70487	17±2.51	14±1.35	11±1.33	5±0.22	ND	ND	12.5
Pichia memberanifaciens CBS107	19±2.41	14±2.64	11±1.11	6±0.36	ND	ND	12.5
Aureobasidium pullulans ATCC42023	18±2.77	13±1.48	10±1.69	5±0.28	ND	ND	12.5
Schizosaccharomyces octosporus EMCC93	18±2.76	15±1.58	12±1.88	7±1.77	ND	ND	12.5
Rhodotorula glutinis EMCC175	17±2.86	14±2.18	11±2.45	6±0.58	ND	ND	12.5
Kluyveromyces Lactis DSM70800	19±1.64	15±2.76	12±2.67	9±1.37	5±0.43	ND	6.2
Hansenula anomala CBS5759	20±2.86	17±1.68	13±2.56	10±2.37	5±0.25	ND	6.2
Candida utilis EMCC41	19±1.84	12±2.78	9±0.34	5±0.36	ND	ND	12.5
Candida albicans EMCC105	23±2.88	15±1.49	6±0.47	ND	ND	ND	25

Data represented are average of triplicates
MIC; Minimum Inhibition Concentration
*Concentrations of extract and MIC are in mg/mL
**Diameter included 5 mm well diameter
ND; Not detected.

Table 6. Inhibition zone diameters and MICs of mix of cana fistula (*Cassia fistula*) and basil (*Ocimum basilicum*) aqueous extracts against bacterial strains

Pathogenic strain	Inhibition zone diameter (mm)**						
	75*	50*	25*	12.5*	6.2*	3.1*	MIC
Gram-positive bacteria							
Bacillus cereus EMCC 1006	33±3.56	30±2.64	25±2.64	20±2.79	15±2.46	ND	6.2
Staphylococcus aureus EMCC1351	31±1.88	28±1.99	24±1.33	16±2.11	9±0.44	ND	6.2
Streptococcus pyogenes EMCC1772	25±0.29	20±1.64	17±2.14	11±0.39	8±0.26	ND	6.2
Streptococcus mutans EMCC1815	28±2.55	22±2.03	18±1.77	13±1.66	8±1.33	ND	6.2
Clostridium perfringens EMCC1574	25±1.97	22±2.55	18±2.77	12±3.13	9±2.50	ND	6.2
Gram-negative bacteria							
Salmonella spp.	30±0.34	24±1.13	12±1.5	9±0.42	5±0.62	ND	6.2
Escherichia coli ATCC25922	32±0.82	29±1.63	22±1.35	18±0.72	9±1.51	ND	6.2
Klebseilla pneumonia ATCC12296	32±1.83	28±0.48	22±1.61	18±2.4	10±0.92	ND	6.2

Data represented are average of triplicates
MIC; Minimum Inhibition Concentration
*Concentrations of extract and MIC are in mg/mL
**Diameter included 5 mm well diameter
ND; Not detected.

Table 7. Inhibition zone diameters and MICs of mix of cana fistula (*Cassia fistula*) and basil (*Ocimum basilicum*) aqueous extracts against yeast and fungi strains

Pathogenic strain	Inhibition zone diameter (mm)**						
	75*	50*	25*	12.5*	6.2*	3.1*	MIC
Yeast							
Saccharomyces cerevisiae DSM70487	32±0.32	29±0.82	23±1.22	17±1.39	11±0.68	5±1.31	3.1
Pichia memberanifaciens CBS107	31±1.83	25±1.44	19±0.76	13±1.87	8±0.36	ND	6.2
Aureobasidium pullulans ATCC42023	33±1.49	28±0.38	24±1.53	18±0.95	12±1.41	6±0.24	3.1
Schizosaccharomyces octosporus EMCC93	32±1.72	27±2.36	24±0.51	19±0.29	15±1.92	8±0.12	3.1
Rhodotorula glutinis EMCC175	25±2.13	21±0.58	17±0.69	13±1.37	9±2.13	5±0.36	3.1
Kluyveromyces Lactis DSM70800	29±0.62	26±1.67	21±1.48	16±0.58	10±1.86	5±0.55	3.1
Hansenula anomala CBS5759	27±0.57	22±1.64	18±0.82	13±1.73	9±1.27	6±0.09	3.1
Candida utilis EMCC41	28±0.37	25±1.39	21±1.83	17±0.98	13±0.18	7±0.37	3.1
Candida albicans EMCC105	33±1.39	29±0.85	24±1.47	19±0.45	11±1.27	ND	6.2
Fungi							
Aspergillusniger EMCC 72	ND	ND	ND	ND	ND	ND	ND
Aspergillusflavus EMCC 274	ND	ND	ND	ND	ND	ND	ND
Aspergillusparasiticus EMCC 886^{T}	ND	ND	ND	ND	ND	ND	ND

Data represented are average of triplicates
MIC; Minimum Inhibition Concentration
*Concentrations of extract and MIC are in mg/mL
**Diameter included 5 mm well diameter
ND; Not detecte.

Similar observation was noticed concerning antifungal activity illustrated in Table 7, where inhibition zone diameters increased to range between (25 – 33 mm) at concentration 75 mg/ mL of the plants mix extract. MICs recorded the lowest value (3.1 mg/ mL) against all tested yeast strains, except for only two strains; *Pichia memberanifaciens* CBS107 and *Candida albicans* EMCC 105 that needed a little higher concentration to be inhibited (6.2 mg/ mL). Generally, many of plant materials used in traditional medicine for their production of secondary metabolites which constitute an important source of micro-biocides and many pharmaceutical drugs. [32] As above mentioned, *Aspergillus* spp. strains did not show any effect when treated with plant mix. *Aspergillus* strains proved to be more resistant in terms of inhibition diameters and MIC, this could be explained by the fact that pathogenicity varies between different strains the higher pathogenicity may exhibit a higher resistance. [33]

3.2. Phytochemical Qualitative Analysis

Table 8 demonstrated phytochemical qualitative analysis of cana fistula (*Cassia fistula*), basil (*Ocimum basilicum*) aqueous extracts and their mix. In both plants, the results indicated the presence of tannins, reducing sugars, flavonoids, volatile oils, amino acids and proteins and absence of glycosides and alkaloids and same results in there mix as well. On the other hand, terpinoides and saponins presence in cana fistula caused its presence in mix, while weak presence of steroids in basil did not affect the mix result, which was negative. A majority of the ascribed biological effects; such as antimicrobial and antioxidant activities of *C. fistula* and *O. basilicum* extracts have been attributed to their primary and secondary metabolite composition. [28,34]

Table 8. Phytochemical qualitative analysis of cana fistula (*Cassia fistula*), basil (*Ocimum basilicum*) aqueous extracts and their mix

Phytochemical	Cana fistula (*Cassia fistula*)	Basil (*Ocimum basilicum*)	Mix
Tannins	+	+	++
Reducing sugars	+	+	++
Glycosides	-	-	-
Alkaloids	-	-	-
Flavonoids	+	+	++
Volatile oils	+	+	++
Amino acids/ Proteins	+	+	++
Terpinoids	+	-	++
Saponins	+	-	++
Steroids	-	+	-

Data represented was confirmed in duplicates
+; for detected, -; for not detected.

3.3. Phenolic and Flavonoids Content

Total phenolic and flavonoids content of cana fistula (*Cassia fistula*), basil (*Ocimum basilicum*) aqueous extracts and their mix are illustrated in Table 9. Total phenolic content (TPC) and total flavonoid content (TFC) of cana fistula aqueous extract was (64.71 and 16.33 mg/g) respectively, which was in agreement with [35]. While TPC and TFC of basil scored (96.42 and 32.66 mg/g) respectively, which exceeded what previously reported by [36,37]. As expected, mix of the two plants recorded higher TPC and TFC (113.07 and 79 mg/g respectively), subsequently, the mix was assayed for its specific phenolic composition through high-performance liquid chromatography before proceeding to antioxidant and antimicrobial impact evaluation.

Table 9. Total phenolic and flavonoids content of canafistula (*Cassia fistula*), basil (*Ocimum basilicum*) aqueous extracts and their mix

Test	Unit	Cana fistula (*Cassia fistula*)	Basil (*Ocimum basilicum*)	Mix
Total phenolic content (TPC)	*mg /g	64.71±1.63	96.42±1.84	113.07±2.46
Total flavonoids content (TFC)	**mg /g	16.33±2.31	32.66±1.67	79±1.38

Data represented in means of duplicates± standard deviation
*Total phenolicwas expressed as Gallic acid equivalents (GAE) mg/ g sample
**Total flavonoidswas expressed as mg catechol/gsample.

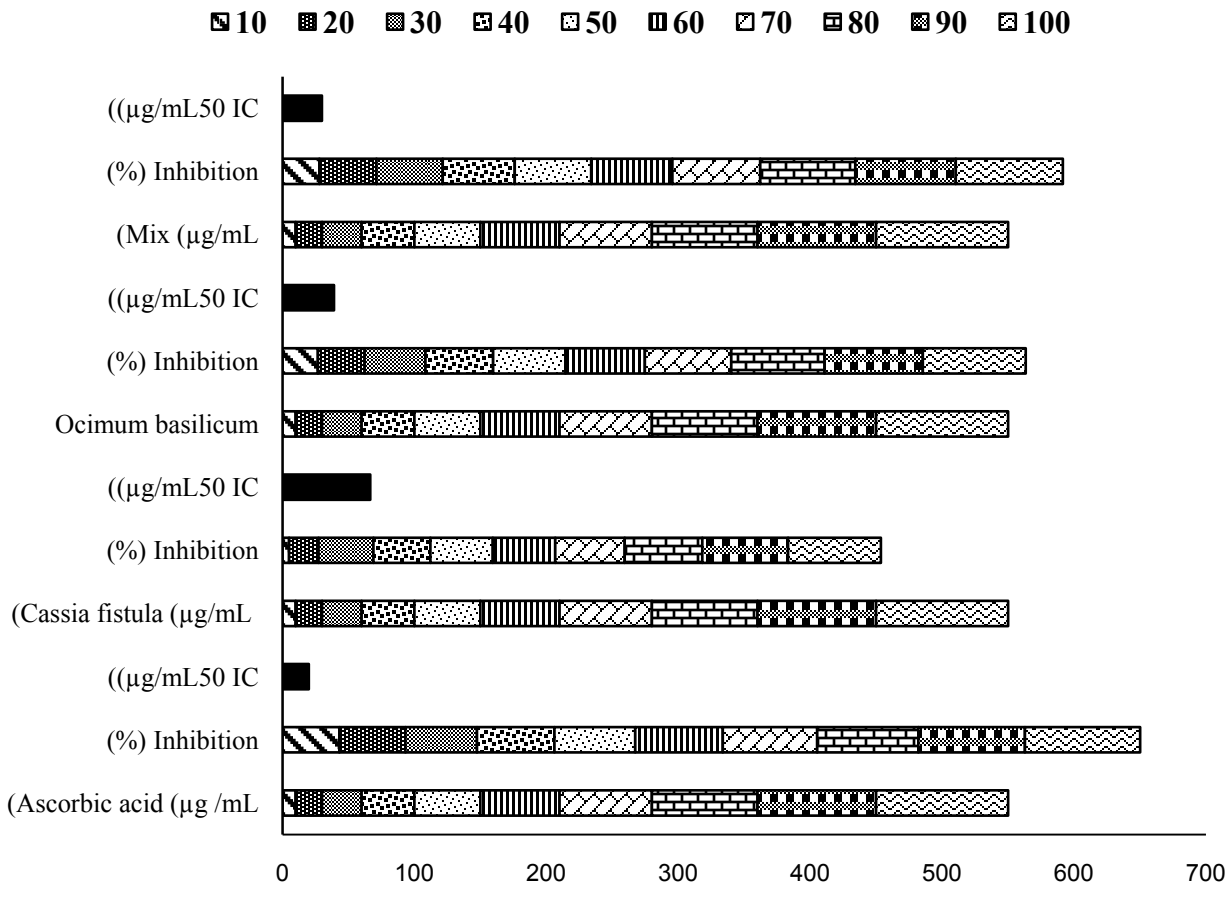
3.4. HPLC Phenolic Compounds Quantification

Table 10 represented phenolic compounds analysis of *C. fistula* and *O. basilicum* mix via HPLC. Amongst nine tested standards, vanillin was > quercetin > coumarin > cinnamic acid which showed the highest concentrations (4.05, 2.53, 2.46 and 2.32 µg/ml respectively), syringic acid and gallic acid recorded less scores (1.42 and 0.32 µg/ml respectively), trace concentrations was recorded by cafeic acid and rutin (0.02 and 0.002 µg/ml respectively), while catechin was not detected. Presence of quercetin, gallic, caffeic and rutin was reported in *C. fistula*. [15,38] On the other hand, caffeic acid and its derivatives such as rosmarinic acid was reported as a strong antioxidant constituents of sweet basil. [28] The extracts are very complex mixtures of many different types of phenolic compounds have different antioxidant activities, which is dependent on their structure [39].

Table 10. Phenolic compounds analysis of canafistula (*Cassia fistula*) and basil (*Ocimum basilicum*) mix via HPLC

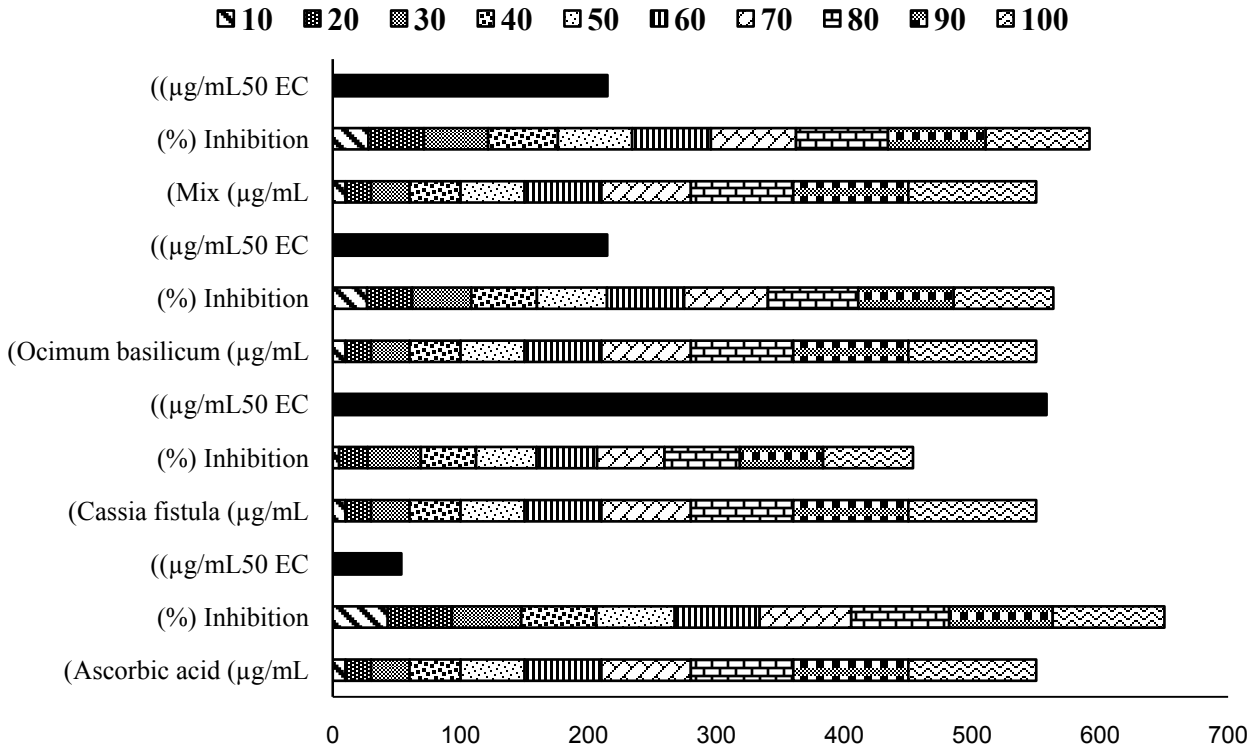
phenolic compound	Concentration(µg/ml)
Gallic acid	0.3242±0.021
Catechin	ND
Syringicacid	1.417±0.24
Caffeic acid	0.0158±0.058
Rutin	0.00156±0.0017
Coumarin	2.456±0.018
Vanillin	4.0464±0.013
Quercetin	2.529±0.26
Cinnamic acid	2.3221±0.026

ND; Not detected.



- Data represented in means of duplicates
- IC50 (µg/ml); Inhibitory concentration at which 50% of DPPH radicals are scavenged.

Figure 1. Antioxidant capacity of different concentrations of cana fistula (*Cassia fistula*), basil (*Ocimum basilicum*) aqueous extracts and their mix using DPPH



- Data represented in means of duplicates
- EC50 (µg/ml); Effective concentration at which the absorbance is 0.5.

Figure 2. Antioxidant capacity of different concentrations of cana fistula (*Cassia fistula*), basil (*Ocimum basilicum*) aqueous extracts and their mix using reducing power

3.5. Antioxidant Activity

Figure 1 illustrated antioxidant capacity of different concentrations of cana fistula, basil aqueous extracts and their mix using DPPH expressed in inhibition % and IC_{50} (µg/ml) (Inhibitory concentration at which 50% of DPPH radicals are scavenged). As can be noticed, ascorbic acid (as a standard), showed the highest inhibition percentage with different concentrations accompanied with the lowest IC_{50} (19.94 µg/mL). On comparing with ascorbic acid results, cana fistula and basil showed less inhibition % with higher IC_{50} (66.51 and 39.03 µg/mL). Obtained cana fistula IC_{50} results are in agreement with [40] while lower values were reported by [41]. Basil obtained IC_{50} results was better than previously reported by [42] who reported higher values. Mixing the two plant aqueous extracts affected positively by arising inhibition % and lowering mix IC_{50} into (29.88 µg/mL). Figure 2 showed antioxidant capacity of different concentrations of cana fistula, basil aqueous extracts and their mix using reducing power expressed in inhibition % and EC_{50} (µg/ml) (Effective concentration at which the absorbance is 0.5). Reducing power results were in harmony with DPPH results. An increase in absorption was exhibited accompanied with the increase in concentrations by ascorbic, both extracts and their mix. Ascorbic acid showed the best inhibition percentage with different concentrations scoring the lowest EC_{50} (53.64 µg/mL). *C. fistula* showed weak inhibition percentages and high EC_{50} (558.03 µg/mL). *O. basilicum* showed better inhibition percentages with EC_{50} (214.59 µg/mL) which was in accordance with [43]. Mixing the two extracts resulted inhibition percent comparable to control and average EC_{50} of (214.77 µg/mL). It is noteworthy that a gradual absorbance increase combined with concentrations is based on its electron donating activity that serves as an important anti-oxidative action [44].

3.6. Cytotoxicity and Anti-inflammatory Assay

Table 11 shows EC_{100} toxic dose of *Cassia fistula*, *Ocimum basilicum* extracts and their mix on human WBCs. It was observed that the EC_{100} was seven-fold higher than EC_{100} of the used anti-inflammatory drug (Hydrocortisone) that reflects the high safety of these extracts. The extracts showed activity to reverse the abnormal stimulation index of LPS-stimulated cells WBCs into normal immune response at 40.6, 255.33, 75.39 and 57.32 µg/ml, respectively. Moreover, *Cassia* extract succeeded to decrease both the inflammatory markers TNF-α and NO., while *Ocimum basilicum* extract reduced these two markers by 77.4% and 72.3%, respectively. Moreover, the mix of the two extract resulted in severe depletion of TNF-α and NO by 85.9% and 76% in respective manner. Based on the obtained date the mix was better than each extract separately. On contrary and as shown in Table 12. COX-2 expression level was up regulated significantly versus control untreated WBCs. Collectively, data presented in Table 11, Table 12 and Table 13 reveal that an extracts of *Cassia fistula*, *Ocimum basilicum* and their mix have high potential activity in suppression the expression of the examined inflammatory markers more than hydrocortisone. Previous studies

reported that *Cassia fistula*, *Ocimum basilicum* and their mix extracts were able to suppress the pro-inflammatory mediators by blocking the activity of the NF-kB which leads to suppression of mitogen-activated protein kinases (p38, JNK and ERK1/2). Blocking of NF-kBwill resulted in inhibiting of TNF-α [45,46] and consequently the expression of COX-2 well influence NO regulation [47,48].

Table 11. EC_{100} toxic dose of *Cassia fistula*, *Ocimum basilicum* extracts and their Mix on humanWBCs

Component	Safe dose EC_{100}(µg/ml)	Effective dose EC (µg/ml)
St. drug (Hydrocortisone)	104.16±4.6	57.32±5.12
Cassia fistula	746.39±8.4	40.67±2.14
Ocimum basilicum	1609.56±38.7	255.33±11.9
Mix	923.27±2.3	75.396±1.27

- Values are expressed as mean±SEM
- EC; Effective concentration.

Table 12. TNF-α (pg/ml) and NO (nmol/ml) levels in extracts- and standard drug-treated LPS-stimulated WBCs compared to LPS-induced WBCs and untreated control WBCs

Component	TNF-α(pg/ml)	NO (nmol/ml)
-veControl (untreated normal cells)	42.61±1.46	29.26±0.93
St. drug (Hydrocortisone)	78.53±1.56	61.4±3.1
Induced	296.57±5.5	100.65±4.01
Cassia fistula	47.87±1.86	36.42±3.75
Ocimum basilicum	67.11±1.9	27.92±0.92
Mix	41.86±1.35	24.083±1.25

- Valuesare expressed as mean ±SEM.

Table 13. Change in level of COX-2 expression in extracts- and standard drug-treated LPS-stimulated WBCs compared to LPS-induced WBCs

Component	COX-2
-ve Control (untreated normal cells)	0.0007±0.00005
St. drug (Hydrocortisone)	0.034±0.00084
Cassia fistula	0.045±0.0047
Ocimum basilicum	0.0053±0.0003
Mix	0.00091±0.000008

Values are expressed as mean ±SEM.

4. Conclusion

In conclusion, *Cassia fistula*, *Ocimum basilicum* extracts and their mix could be recommended as natural food preservatives due to their obvious role in food borne pathogen control as biological food contaminants, in addition to their antioxidant and anti-inflammatory activities. Obtained results can qualify examined extracts to become promising in safe food production protecting human from food-born pathogens with absence of abnormal inflammation or immune responses.

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