In vitro assessment of anti-inflammatory and COX-2 inhibitory action of some medicinal plants

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Abstract

Objective of this research was to evaluate the anti-inflammatory and COX-2 inhibitory properties of celery, myrrh, aqueous and alcoholic fenugreek plant extracts.

The Human Red Blood Cell membrane stabilization (HRBC) method was used to evaluate the anti-inflammatory effect and the Enzyme Immunoassay (EIA) was used to evaluate the COX-2 inhibitory actions.

The celery extract showed excellent anti-inflammatory and COX-2 inhibition potency. The other extracts (myrrh and fenugreek) showed less activity that might indicate high efficacy of celery. This may be attributed to the plant constituents, mainly to a compound known as 3-n-butylphthalide, which is the most powerful of all healing factors of celery.

Introduction

Celery is the common name of *Apium graveolens* L., family Apiaceae. It originates in the Mediterranean area, but it also grows in salty soils of North Africa, South America, Europe, Asia, and Africa. It is source of potassium, folic acid, phosphorus, calcium, dietary fibres, vitamin A, vitamin B-complexes, vitamin C, vitamin E, and a compound known as a 3-n-butylphthalide (3nB).[1] Traditional medicine uses of celery are rheumatic tendencies, gout, flatulence, chronic pulmonary catarrh, tendencies toward overweight, lack of appetite, skin problem, bronchitis, and rheumatism. Celery is also used as a urinary antiseptic because it has strong diuretic effect. On the other hand, celery should not be used when a person suffers from acute kidney problems. Furthermore, celery seeds are used to treating anaemia and arthritis.[1,2]

Fenugreek *Trigonella foenumgræcum* L., family Fabaceae, has been used for very long time as medicinal and nutrient herbs. The fenugreek seed contains 45-60% carbohydrates (mainly galactomannans 20-30%), 5-10% proteins and 5% fixed oils (lipids). Moreover, it also contains pyridine type alkaloids, mainly trigonelline (0.2-0.36%), choline (0.5%), gentianine and carpaine. The fenugreek seed also contains some flavonoids such as apigenin, quercetin, vitexin, and isovitexin. It has some minerals like calcium, and iron; vitamins A, B_1, C, and nicotinic acid.[2] Fenugreek showed good therapeutic effects to treat cancer, scraggy, inflammation, diabetes mellitus, cataract, and gastric disorders.[3]

Myrrh gum is the popular name of *Commiphora molmol* L. Engler, family Burseraceae. Myrrh contains water-soluble polysaccharides, proteins, alcohol-soluble resins, and volatile oils. It has been used for dyspepsia, gums problems and asthma.[4,5] The antibacterial and anti-inflammatory properties of myrrh have not been explored over a wide range.[6]

Inflammation has a major function for various pathophysiological cells in extracellular fluid. It is a protective mechanism against contamination, damage, or injury.[7] The inflammation course in cooperation with the macrophages provide intermediates against external materials. Macrophages produce immediately in the activation level proinflammation mediators such as Prostaglandin E_2 (PG-E_2), leukotrienes, Interleukin (IL), Nitric Oxide (NO), and Tumour Necrosis Factor-α (TNF-α).[7,8] In addition to inflammation, the inflammatory mediators are also produced more frequently in several diseases like atherosclerosis, rheumatoid arthritis, asthma, and pulmonary fibrosis.[8]

Cyclooxygenase (COX), officially known as prostaglandin-endoperoxide synthase, is the most important enzyme among proinflammation mediators. This enzyme has at least three isoforms: COX-1, COX-2, and COX-3. The COX enzymes are inhibited directly by non-steroidal anti-inflammatory drugs (NSAIDs).[9] The NSAIDs that inhibited COX-2 had fewer side effects.[10] The COX-2 enzyme concentration is amplified in several cancer types, e. g. colon, breast or lung malignancy. Medicinal plants have been studied extensively as anti-inflammatory agents, but the selectivity of these plants toward one of the COX isoforms, especially COX-2, is lightly studied.[11]

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In this research, plants celery (*Apium graveolens* L.), fenugreek (*Trigonella foenumgraecum* L.), and myrrh (*Commiphora molmol* L.) were studied as selective COX-2 inhibitors.

Materials and Methods

Chemicals

All chemicals used in this research were analytical grade. Ethanol, iodine, picric acid, β-naphthol, sulphuric acid, and dimethyl sulfoxide (DMSO) were supplied from Sigma-Aldrich Company (Missouri, MO, USA). Enzymes Immunoassay (EIA) kit (Catalogue No. 560131) was supplied from, Cayman Chemical, (Ann Arbor, Michigan, MI, USA).

Preparation of plant extracts

Plant powder (20g) of each plant [Celery Seeds (CS), Fenugreek Aqueous seeds (FA), fenugreek seeds alcoholic (FL) and Myrrh Gum (MG)] was added to 100 ml of distilled water, then the mixture was refluxed for 3hr. Also, fenugreek seeds powder 20 g was added to 100 ml of ethanol 96%, then the mixture was refluxed for 3 hr. to prepare fenugreek seeds alcoholic extract. The extracts were tested for the presence of phytochemicals as follows: for the detection of alkaloids, 1 ml of extract was treated with 1 ml of Wagner's reagent (Iodine in potassium iodide solution); the formation of a brown/reddish brown precipitate indicates the presence of alkaloids. Likewise, 1 ml of extract treated with 1 ml of Hager's test (saturated picric acid solution), the formation of a yellow colour precipitate indicated the presence of alkaloids.[12] On the other hand, the detection of carbohydrates was carried out using Molisch's test: 2 ml of extract treated with 5 drops of alcoholic β-naphthol solution 2% and 2 ml of concentrated sulphuric acid were added carefully along the sides of the test tube. The formation of the violet ring at the junction indicates the presence of carbohydrates and glycosides. The extracts were dried to powder by using rotary evaporator to be used in anti-inflammatory activity and COX-2 inhibitory activity assay.[13]

In vitro anti-inflammatory activity

Human red blood cells (HRBC) method was used to estimate *in vitro* anti-inflammatory activity. The solutions used in this method are: i) Alsever's solution which was prepared dissolving 2.05% glucose, 0.41% NaCl, 0.81% trisodium citrate and 0.056% citric acid, in distilled water to final volume of 100 ml; ii) Hyposaline (0.7% NaCl); iii) Isosaline (0.9% NaCl); and iv) Phosphate buffer (pH 7.4). Blood (5 ml) was collected from a healthy volunteer and mixed with an equal volume of sterilized Alsever's solution.[14] The serum was obtained by centrifugation at 4000 rpm for 12 min. and the red blood cells (packed cells) were separated. The HRBC suspension was prepared by washing packed cells with isosaline solution (3 ml x 2), then the volume was adjusted to 10 ml with isosaline solution; 50, 100, and 200 mg of celery, myrrh, and fenugreek extracts were used. The dosages were dissolved in 1 ml of distilled water for the aqueous extracts, while the alcoholic fenugreek extract was dissolved in 1 ml of 95% ethanol. Samples of each plant extract, control and standard (indomethacin, at a dose 50, 75, 100 mg) were separately mixed with 1 ml of phosphate buffer, 2 ml of hyposaline, and 0.5 ml of HRBC suspension. The assay mixtures were incubated at 36.5 ºC for 30 min. in an oven and then they were centrifuged at 3000 rpm for 10 min. The supernatant was decanted, and haemoglobin content was estimated by spectrophotometer at 560 nm wavelength. The percentages of haemolysis were estimated by assuming the haemolysis produced in the control as 100%, according to the following equation.[15]

$$\text{Percentage protection} = 100 - \left(\frac{\text{Absorbance of Sample}}{\text{Absorbance of Control}}\right)$$

In vitro assay of COX-2 inhibitory activity

In vitro COX-2 inhibition potency was estimated by Enzyme Immunoassay (EIA) method. EIA kit was used to examine the plant extracts activity toward COX-2 inhibition. The plant extracts were dissolved in 1 ml of DMSO (99%) in different amounts (50, 100, and 200 µg/ml), to estimate the inhibition activities according to the manufacturer's protocol.[16] Indomethacin was used as a positive control in dosages (50, 75, and 100 µg/ml); 10 µl of plant extracts and indomethacin dosages were added to the reagents from EIA kit, 960 µl reaction buffer solution, 10 µl COX-2 enzyme and 10 µl heme. Then the solutions were incubated for 10 min at 36.5 ºC, after that 10 µl of Arachidonic Acid (AA) were added: immediately afterwards 50 µl of 1 M HCl was added to finish the COX-2 reaction. Stannous chloride (100µl) was added to convert prostaglandin H2 (PG-H_2) to prostaglandin F2α (PG-$F_{2\alpha}$) via reduction reaction, the COX-2 enzyme catalysed reaction of arachidonic acid to produce PG-H_2.[17]

When the solutions color became yellow, and the concentration of the PG-$F_{2\alpha}$ was estimated spectrophotometrically using UV-vis spectrophotometer at 418 nm. The percent of inhibition was calculated by comparison of extracts measurements with control assessment.[18]

Statistical analysis

The results are represented as mean ± Standard Error (SE), the samples were done with three replicates (n=3). Statistical analysis was carried out by analyses of variance for one way (ANOVA), The probability (p) was considered significant if the value of p is less than 0.05.

Results

Table 1 shows the alkaloids, carbohydrates, and glycosides qualitative tests of extracts. Fenugreek contains alkaloids, carbohydrates, and glycosides in both aqueous and alcoholic extracts. Myrrh and celery do not contain alkaloids, but they contain carbohydrate and glycosides. Table 2 displays the results of the human red blood cell membrane protection percentage. CS and MG showed a significant effect ($p<0.05$) and protection percent (81.3±6.2 and 78.7±4.9, respectively at concentration 200 µg/ml). FA and FL did not show significant data, compared with positive control protection percentage (92.1±3.1 at concentration 100 µg/ml). Table 3 shows the inhibition percent of the extracts on COX-2; CS and MG gave significant results ($p<0.05$) and inhibition percent (74.1 ± 4.6 and 72.5 ± 5.6, respectively at concentration 200 µg/ml). However, FA and FL did not show a significant effect. Figures 1 to 5 explain the results of COX-2 inhibitory activity. The half-maximal inhibitory concentration (IC_{50}) for the extracts and standard drug (indomethacin) were calculated from Figures 1 to 5; positive control indomethacin showed IC_{50} = 40 µg/ml (Figure 1). Figures 2 and 3 show better COX-2 inhibition compared to control for CS and MG, with IC_{50} = 60 and 65 µg/ml, respectively. Figures 4 and 5 show less COX-2 inhibition activity than the control for FA and FL with IC_{50} = 149 and 93 µg/ml, respectively.

Table1. Qualitative tests results.

Plant Tests	Fenugreek aqueous extract	Fenugreek alcoholic extract	Myrrh	Celery
Wagner's test	+	+	-	-
Hager's test	+	++	-	-
Molisch's test	++	++	+	++

Table 2. Statistical analyses of the calculated data for inflammation protection percentage.

Sample	Concentration (µg/ml)	Percentage protection (%)
Celery (CS)	50	51.4±7.1
	100	71.8±4.3*
	200	81.3± 6.2*
Fenugreek aqueous (FA)	50	15.3±4.6
	100	43.6±5.4
	200	61.4±3.6
Fenugreek alcoholic (FL)	50	21.6±8.3
	100	51.8±5.8
	200	56.9±6.5
Myrrh (MG)	50	48.9±6.3
	100	71.3±3.4
	200	78.7±4.9*
Indomethacin	50	58.4±2.7
	75	80.6±3.9*
	100	92.1± 3.1*

Note: The values are mean±standard error, *mean p<0.05.

Table 3. COX-2 inhibitory activity of plant extracts.

Sample	Concentration (µg/ml)	Inhibition Percentage (%)
Celery (CS)	50	46.3±5.7
	100	65.9±2.7
	200	74.1±4.6*
Fenugreek Aqueous (FA)	50	17.5 ± 4.8
	100	41.3 ± 6.2
	200	59.2±4.1
Fenugreek alcoholic (FL)	50	19.8±6.3
	100	55.0±4.4
	200	57.9±4.7
Myrrh (MG)	50	45.2±3.2
	100	61.7±5.4
	200	72.5±5.6*
Indomethacin	50	56.2±3.2
	75	67.5±5.5
	100	78.3±4.3*

Note: The values are mean±standard error, * mean p<0.05.

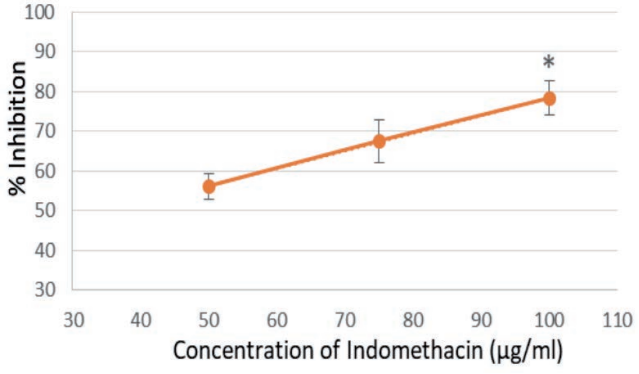

Figure 1. COX-2 inhibitory activity of indomethacin.

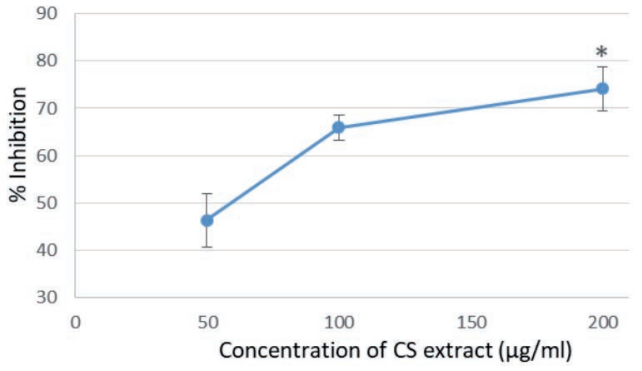

Figure 2. COX-2 inhibitory activity of CS.

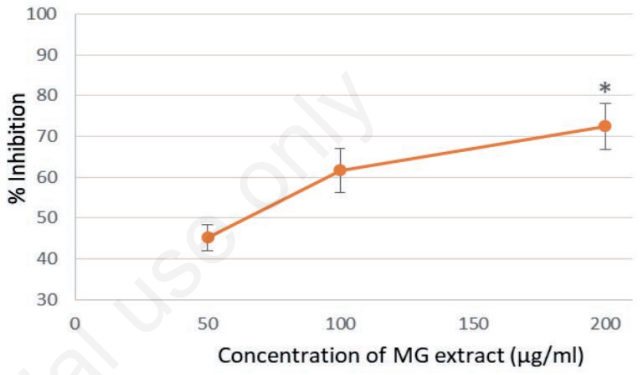

Figure 3. COX-2 inhibitory activity of MG.

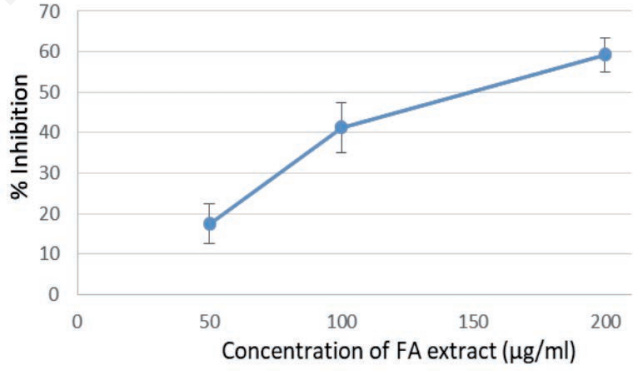

Figure 4. COX-2 inhibitory activity of FA.

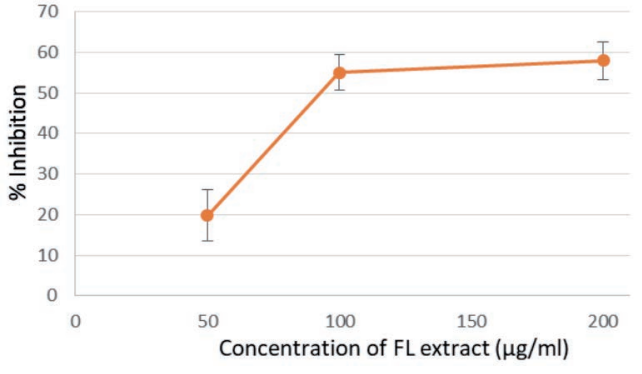

Figure 5. COX-2 inhibitory activity of FL.

Discussion

Inflammation is a common phenomenon which includes several reactions of living tissues towards injury. When anti-inflammatory drugs are frequently used, especially steroidal anti-inflammatory drugs, the risk of side effects of these drugs increases. Moreover, steroidal anti-inflammatory agents will lyse and possibly induce the redistribution of lymphocytes, which causes a rapid and transient decrease in peripheral blood lymphocyte counts to affect long-term response.[19]

HRBC method was selected for the *in vitro* evaluation of anti-inflammatory property because the erythrocyte membrane is analogous to the lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membranes.[20] The equilibrium of lysosomal membrane is a key factor for regulating the inflammatory reaction by stopping the release of lysosomal ingredients of neutrophil, such as enzymes among which proteases, which cause destruction upon release.[21] The COX-2 inhibition results indicate that the plant extracts of celery, myrrh, and fenugreek have significant anti-inflammatory properties ($p< 0.05$). These significant anti-inflammatory properties may be due to the inhibition of any inflammatory mediators by the glycosides or alkaloids present in the extract. Also, celery and myrrh show good COX-2 inhibition activity in comparison to the control (indomethacin). The compound 3-n-butylphthalide (3nB) might be the cause of celery extract COX-2 inhibition efficacy. Many researchers showed 3nB, which is recognized in celery, as a violent healing factor, antioxidant, preventing coagulation, aggregation of platelets, lowering of blood pressure, and lipids.[22,23] Therefore, celery extract can be useful to humans, and it can be given with known anti-inflammatory drugs, because presumptively it does not interact with these medicines.[23,24]

Conclusions

Inflammation is a reactions cascade which can lead to something tissue harm. It is characterised by increased blood flow to the tissue causing increased temperature, redness, swelling, and pain. Numerous scientific studies have looked for new anti-inflammatory substances, which are more selective and have fewer side effects. The plant extracts used in this study are from celery, myrrh, aqueous and alcoholic fenugreek. The present results indicate the efficacy of celery over other plants; this may be due to the plant constituents, particularly, to a compound known as 3-n-butylphthalide (3nB), as the scientists suggest.[23] This is augmented by the increasing tendencies towards evaluating the most powerful of the healing factors (3nB).[24] In conclusion, there is a need to investigate (3nB) activity towards COX selectivity using *in vivo* study.

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