The use of ultrasound (UAE) and microwaves (MAE) to improve the extraction of pharmaceutically active materials from the fruit of the prickly pear (Opuntia ficus-indica)

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Abstract

Background

Conventional extraction methods have many limitations and drawbacks, including, prolonged extraction time, present safety concern and environmental risks, with low quality extracts. We report the use of ultrasonic (UAE) and microwave (MAE) assisted technologies as techniques for improving the extraction of pharmaceutically active materials from *Opuntia ficus-indica* (OFI) a species of prickly pear.

Results

The pulp and peel of the plant fruit were used and the total phenolic and flavonoid content were evaluated. Antioxidant assay (DPPH), was employed to prescreen different extracts. The Hepa1c1c7 model was used for testing the induction of chemopreventive marker protein NAD (P) H-quinone oxidoreductase 1 (NQO1). *In vitro* anti-inflammatory activity was performed on RAW 264.7 macrophage model induced for nitric oxide (NO) release in the presence of lipopolysaccharide. *In vivo* study included testing the therapeutic potential of some extracts on carrageenan induced paw edema in adult rats. Our data showed that fruit peels had the highest contents of phenolic and flavonoid compounds of OFI extracted using microwave assisted extraction (MAE) at 800 W power for 5 min extraction time **(EXM1)** with a percentage increase of 74.1% and 115.3% respectively, when compared to conventional maceration. The fruit pulp showed the highest phenolic and flavonoid content using MAE at 400 W power for 15 min extraction time **(EXM2)** with a percentage increase of 55.4% and 105.8% respectively, when compared to conventional maceration. DPPH prescreen revealed the potency of the **EXM1** among other tested extracts, recording EC_{50} 148 µg/ml. Although *in vitro* chemopreventive as anti-inflammatory model revealed no activity on (NQO1) induction of **EXM1**, the *in vivo* model gave positive results. Edema size reduction percentage of **EXM1** was 104% after 4 hours. Anti-inflammatory markers indicated that **EXM1** inhibited COX-2, IL-6, TNF-α, and TGF-β1 more significantly than indomethacin and conventional extraction methods, while inhibition levels of NO and MDA in case of **EXM2** extract were more significantly than other extraction methods. Moreover, extracts obtained using MAE showed a significant increase in antioxidant enzymes (GSH and SOD) than those obtained using conventional method.

Conclusions

The data obtained further confirming the beneficial value of MAE and UAE technologies for extraction of the active material of (OFI).

1. Background

Opuntia ficus-indica (L.) (OFI) Mill. belongs to the Cactaceae family with the greatest economic relevance in the world [1]. It has been a domesticated plant in Latin America, Africa, Mediterranean countries, the

Middle East, Egypt, India and Australia [2, 3]. It is a medicinal herb [4], containing mixtures of bioactive compounds whose profiles change with species, cultivars and climate conditions [5]. It has been used as food source for animals and human [6]. It is a relevant source of phytochemicals with proven biological activities and high added value in the food/ nutraceutical industry [7]. There is an increasing usage of fresh fruit to produce several products such as juice, jelly …etc.[8].
It has been reported that (OFI) contain polyphenolic compounds such as flavonoids and phenolic acids [9, 10]. Polyphenols are an important group of compounds associated with (OFI) fruits which were found to possess antioxidant, anti-inflammatory activity [11] and antimicrobial properties [12].

Opuntia fruit/pulp extracts possess antidiabetic [13], cardioprotective, neuroprotective [14], anti-inflammatory [15] and hepatoprotective properties [16].

In biological system, oxidative stress rises after an increased contact to oxidants, a reduction of anti-oxidant total capability in the system. It is frequently related to ROS generation and free radicals [17].

A cellular damage is caused from this oxidative stress and leads to many diseases like cardio vascular disease and cancer [18]. Antioxidants protect cells against ROS molecules and help organisms to deal with oxidative stress, caused by free radical such as a- Enzymatic antioxidant defenses including catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD). b-Non-enzymatic antioxidants including glutathione (GSH), flavonoids and carotenoids [19].

The natural products such as herbs have inroad powerful antioxidants and anti-inflammatory to inhibit the process of cellular transformation. Also, nitric oxide (NO) is presented as a critical mediator in the inflammation process which is produced by iNOS at site of inflammation [20].

Agents such as TGF-ß and IL-10 are involved in the down regulation of inflammatory reactions [21]. Cytokines are categorized as pro-inflammatory IL-6 and TNF- α as well as the inflammatory-cytokines IL-10 and TGF-ß produced predominantly by activated macrophages, involved in the up-regulation of inflammatory reactions [22]. COX enzymes are the main enzymes responsible for prostaglandin biosynthesis. The modulation of the enzymes can assist in anti-inflammatory treatments owing to the key role of PG especially PGE2 in the inflammatory response [23].

Nowadays, extraction development of polyphenolic components from Opuntia spp. has a great attention among researchers. This could be contributed to Opuntia spp. oxidant/anti-oxidiant potential [24].

In the last years, efforts have been oriented to exploiting new extraction methods, such as ultrasonic assisted extraction (UAE) [25] [26], microwave assisted extraction (MAE) [27], pressurized liquid extraction (PLE) [28] and supercritical fluid extraction [29], which have emerged as energy-saving technologies.

In addition to its economic impact, food and nutraceutical industry prefers green extraction process to obtain high-quality products [30].

The extraction technique based on ultrasonic waves was utilized to reduce solvent consumption and extraction time and to obtain better yields [31]. The UAE mechanism is attributed to the mechanical and cavitation effects, which increase mass transfer of targeted compounds due to cell wall breakage of plant material [32], ultrasonic waves also ease the extraction of the solute from inside the plant matrix to the extraction solvent at faster rate [33] [34] [35].

Another green extraction technique is microwave assisted extraction (MAE). This technique involves irradiation of the samples which soaked in a solvent. Furthermore, comparing to conventional extraction methods (oil baths, sand baths and heating mantles) that cause thermal decomposition of substrates, microwaves can pass through the walls of the reaction container, thereby heating directly the reactants and the solvent [36].

Microwave assisted extraction is utilized on a large laboratory scale because it has numerous advantages like reducing energy consumption as well as the amount of organic solvents, reducing waste and makes possible to obtain better yield with respect to the conventional methods of extraction [37]. The extraction efficiency is reliant on several factors, such as nature of the compounds to be extracted, temperature, pH, nature and volume of solvents.

In several published articles, comparing microwave assisted extraction with other innovative and conventional extraction methods, microwave assisted extraction has been known as a potential and powerful alternative for the extraction of bioactive components from herbal material [38].

For the above reasons it is essential to select the suitable method for the extraction of bioactive compounds, one which aids to reserve their antioxidant properties. The current study has examined the effect of two advanced green techniques on chemical content of (OFI) different parts extracts, and their influence on their biological activity.

2. Results

2.1|Estimation of total phenolic and flavonoids of different (OFI) extracts:

The current study indicated that phenolic and flavonoid content of OFI peel different extracts were highly significant ($P < 0.01$) compared to pulp extracts. Peel MAE extract at W 800 after 5 min extraction **(EXM1)** showed highest content of both phenolic and flavonoid among all extracts.

2.1.1|Effect of UAE on the amount of TPC and TFC extracted from (OFI) different parts

During our study a significant difference in the total amount of phenolic and flavonoid contents of peel and pulp belonging to (OFI) was observed. In general, (OFI) methanolic peel extract showed higher contents of both phenolic and flavonoid contents. A slight increase in TPC and TFC from (OFI) peel **EXC1** [1880 µg/ml GAE 100mg extract and 480 µg/ml RE 100mg extract, respectively] and pulp **EXC2** [433 µg/ml GAE 100mg extract and 57 µg/ml RE 100mg extract, respectively] was observed after increasing the conventional maceration extraction time from 15 to 30 mins.

We examined the effect of both ultrasonic waves and duration of extraction on extractability of TPC and TFC from (OFI) peel and pulp. In case of (OFI) pulp and peel, a significant increase in the extraction efficiency was observed on TPC and TFC using direct sonication (20 Khz) when compared to silence extraction as shown in Fig. (1) and (2).

2.1.2|Effect of MAE on the amount of TPC and TFC extracted from (OFI) different parts

Extractability of TPC and TFC from (OFI) peel and pulp using microwave assisted extraction increased significantly when compared to conventional maceration as shown in Fig. (1) and (2).

The highest TPC and TFC from (OFI) peel were observed after 5 mins MW irradiation at 800 W **(EXM1)** [2740 µg/ml GAE 100mg extract and 610 µg/ml RE 100mg extract, respectively], whereas, the highest TPC and TFC from (OFI) pulp were observed after 15 mins MW irradiation at 400 W **(EXM2)** [626.66 µg/ml GAE 100mg extract and 93.33 µg/ml RE 100mg extract, respectively].

2.2|Estimation of in-vitro antioxidant activity for different (OFI) extracts by using DPPH assay (Radical scavenging activity):

The DPPH is a stable free radical used for the study of structural characteristics to the radical scavenging activity of compounds. As shown in Fig. (3a). The preliminary screening of OFI peel and pulp extracts (EXM1, EXC1, EXU1, EXM2, EXC2, EXU2) showed that **EXM1** had the highest radical scavenging potency among the tested extracts compared to rutin reference radical scavengers by 90.8%. Concentration-response experiment showed a gradual increase of the DPPH radical activity with increasing concentration. Effective concentration that scavenges 50% of DPPH (EC_{50}) in the vehicle control was calculated on GraphPad prism software to be 148 µg/ml Fig. (3b). In the present study **EXM1** had the highest DPPH activity recorded among other extracts.

2.3|Estimation of in-vitro anti-inflammatory activity for EXM1:

The NO inhibition assay as employed to assess the inhibition of LPS induce NO from culture RAW macrophage. **EXM1** showed a very weak inhibition of NO release Fig. (4).

2.4|Estimation of in-vitro chemopreventive activity for EXM1 at different concentration:

Assessment of the cancer chemopreventive potential by Western blot analysis of the NQO1 protein expression for **EXM1** showed no effect within the concentration rang tested (6.25, 25 and100 µg/ml) as shown in Fig. (5).

2.5|Toxicity in vivo studies of different (OFI) extracts:

After 24h, the behavior pattern of mice were tracked upon applying the four (OFI) extracts at different doses: 7, 6, 5, 4, 3, 2 and 1g/kg body weight. Treated animals did not show any marked changes. The median lethal dose LD_{50} for promising extracts were illustrated in tables: 1, 2, 3 and 4.

Results obtained from table 1, 2, 3 and 4, the treated doses of EXM1, EXC1, EXM2 and EXC2 extracts which used in carrageenan induced paw edema model were 1/10 LD_{50} as follows 400, 80, 120 and 120 mg/kg, respectively.

Table (1): Acute toxicity of EXM1 extract.

Dose (g / kg b.wt.)	Mice number	Dead mice number	Z	d	(Z)x(d)
7	6	6	6	1	6
6	6	5	5.5	1	5.5
5	6	3	4	1	4
4	6	1	2	1	2
3	6	0	0.5	1	0.5
2	6	0	0	1	0
1	6	0	0	1	0
LD_{50} of EXM1 = 4g/kg b. wt.					

Table (2): Acute toxicity of EXC1 extract.

Dose (g / kg b.wt.)	Mice number	Dead mice number	Z	d	(Z)x(d)
7	6	6	6	1	6
6	6	6	6	1	6
5	6	6	6	1	6
4	6	3	4.5	1	4.5
3	6	1	2	1	2
2	6	0	0.5	1	0.5
1	6	0	0	1	0
LD_{50} of EXC1 = 0.8g/kg b. wt.					

Table (3): Acute toxicity of EXM2 extract

Dose (g / kg b.wt.)	Mice number	Dead mice number	Z	D	(Z)x(d)
7	6	6	6	1	6
6	6	6	6	1	6
5	6	6	6	1	6
4	6	2	4	1	4
3	6	0	1	1	1
2	6	0	0	1	0
1	6	0	0	1	0
LD_{50} of EXM2 = 120g/kg b. wt.					

Table (4): Acute toxicity of EXC2 extract.

Dose (g / kg b.wt.)	Mice number	Dead mice number	Z	D	(Z)x(d)
7	6	6	6	1	6
6	6	6	6	1	6
5	6	6	6	1	6
4	6	2	4	1	4
3	6	0	1	1	1
2	6	0	0	1	0
1	6	0	0	1	0
LD_{50} of EXC2 = 120g/kg b. wt.					

2.6| Biological in-vivo anti-inflammatory and antioxidant studies for different (OFI) extracts:

2.6.1| Estimation of rat paw edema curve:

Data obtained from (Fig. 6) indicated that **EXM1** extract **(G5)** showed highly significant ($P < 0.01$) inhibition of edema size, with percentage inhibition 89.2% after 3 hours and 104% after 4 hours, followed by **EXM2** extract **(G7)** with percentage inhibition 69.6% after 3hours and 94.6% after 4 hours, when compared to G2 (CARR group). A decrease in inhibition of edema size percentage was obserbed by exracts obtained by convinationl methods, **EXC1** extract (G4) and **EXC2** (G6) showed inhibition by 42% and 58% after 4 hours, respectively.

2.6.2| Estimation of antioxidant enzymes (MDA, NO, SOD and GSH) and anti-inflammatory markers (COX-2, IL-6, TNF-α, and TGF-β1) in serum samples

The current study was performed to investigate the anti-inflammatory activity of (OFI) different extracts against carrageenan-induced rat paw edema. The activities of these extracts were directly correlated to their constituents as antioxidant agents (phenolic and flavonoid contents) and enhanced using advanced green technology Fig. (7).

In the current study, it was indicated from the results that GSH levels were significantly decreased ($p < 0.05$) in PCT (G2) compared to NCT (G1). GSH levels were significantly increased in G3, G5 and G7 compared to PCT (G2) (Fig. 7A). In addition, SOD levels were significantly decreased ($p < 0.05$) in PCT (G2) compared to NCT (G1). SOD levels were significantly increased ($p < 0.05$) in G5 and G7 compared to PCT (G2) (Fig. 7A).

Furthermore, NO and MDA levels were significantly increased ($p < 0.05$) in PCT (G2) compared to NCT (G1). Moreover, NO and MDA levels were significantly decreased ($p < 0.05$) in G5, and G7 ($p < 0.01$) compared to PCT (G2) (Fig. 7B). Our findings also indicated that cytokines; IL-6, TNF-α, and TGF-β1 levels were significantly increased ($p < 0.05$) in PCT (G2) compared to NCT (G1) and significantly decreased ($p < 0.05$) in G5, and G7 compared to PCT (G2), while G4 and G6 showed significant increase ($p < 0.05$) in IL-6, TNF-α, and TGF-β1 levels compared to PCT (G2) (Fig. 7C). Finally, COX-2 levels were significantly increased ($p < 0.05$) in PCT (G2) compared to NCT (G1). Also, G3, G4, and G6 showed significantly increased in COX-2 levels compared to PCT (G2), especially G4 and G6 which showed high significance increase ($p < 0.01$), while G5 and G7 showed significantly decreased in COX-2 levels ($p < 0.05$) compared to PCT (G2) (Fig. 7D).

3. Discussion

Effect of UAE on the amount of TPC and TFC extracted from (OFI) different parts

The unique mechanism offered by ultrasonic waves, which depends mainly on the cavitations phenomena [39], increased the extractability of TPC and TFC from (OFI) different parts. The highest TPC and TFC from (OFI) peel and pulp were observed after 15 mins sonication **EXU1** [2180 µg/ml GAE 100mg extract and 493 µg/ml RE 100mg extract, respectively] and **EXU2** [500 µg/ml GAE 100mg extract and 66.67 µg/ml RE 100mg extract, respectively]. The extraction yield is elevated significantly due to the formation of several high speed jets produced as a result of ultrasonic cavitations phenomena, which are able to strike the cell walls and increase the mass transfer from inside the cells of the vegetal material to the surrounding extraction solvent. However, a slight decrease was observed at 30 mins extraction time using UAE as increasing the sonication time can cause degradation/break down of some compounds [40].

Effect of MAE on the amount of TPC and TFC extracted from (OFI) different parts

Microwave distinctive heating mechanism does not only increase the penetration force of the solvent to the cell wall of the plant matrix, but it also causes evaporation to the moisture content inside plant cells and generation of enormous pressure. The produced pressure pushes the cell wall from inside and ultimately rupturing it, which facilitates the complete rupture of the plant cell and leaching out of the active constituents from the ruptured cells to the extraction solvent, thus improving the yield of phytoconstituents [27]. In addition, microwave increases the number of plant cells pores, which facilitates the complete release of the bioactive principles to the extraction solvent. Moreover, MAE increases the extraction selectivity of phenolic compounds when compared to conventional methods. Phenolic compounds molecules are characterized by dipole moment. These compounds strongly absorb microwave energy, and consequently it is considered one of the most successful MAE applications among all other classes of compounds [41, 42] reported an increase in the extractability of bioactive phenolic compounds about four times higher than other extraction methods. [43] and [44] reported significant increase in the extraction efficiency of phenolic compounds using microwave energy from different herbal plants. The power used during MAE and the structure of the plant matrix under treatment have great effect on the extraction efficiency of bioactive compounds. In our study, extraction of (OFI) pulp using 400 W microwave irradiation showed better results when compared to 800 W at all time intervals. The physiological composition of the pulp is characterized by softer structure (more juice/water) than the peel, which requires less drastic conditions of extraction to prevent the degradation of the extracted compounds. Contrastingly, extraction of (OFI) peel needed more powerful extraction conditions, using 800 W microwave irradiation power for 5 mins, due to the physiological composition of the peel which is characterized by higher fiber content [1]. However, a slight decrease in the total phenolic content of (OFI) peel was observed after prolonged extraction under drastic condition, due to the possible degradation of the target compounds. A similar behavior was observed by [45].

Estimation of in-vitro **antioxidant activity for different (OFI) extracts by using DPPH assay (Radical scavenging activity)**:

The DPPH is a stable free radical. In the present study **EXM1** had the highest DPPH activity recorded among other extracts. The obtained activity correlates well with the total phenolic and flavonoid content of this extract which indicated the influence of phenolic constituents on antioxidant capacity. In consistent with our findings same previous studies reported the (DPPH) activity [46], [47], [48], [49], [18] and [50].

Estimation of antioxidant enzymes (MDA, NO, SOD and GSH) and anti-inflammatory markers (COX-2, IL-6, TNF-α, and TGF-β1) in serum samples

In the light of the strong interconnection between inflammations and redox-modulating properties of phytochemicals plant, phenolic and flavonoid content play an important role either as antioxidant or anti-inflammatory agent with different mechanisms. For example, the biological system depends on numerous endogenous defense mechanisms to protect against cell damage induced by free radical through the antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH)

which offer the cellular first line defense against toxic free radicals. The major role of GSH is to detoxify the radicals such as NO an MDA through scavenging them or by acting as a co-substrate in the glutathione peroxidase (GPx)-catalyzed reduction of lipid peroxides and hydrogen peroxide. SOD activity protects cells and the extracellular matrix from the harmful effects of superoxide anion and its derivatives such as hydroxyl radical [51].

In acute inflammation as carrageenan induced paw edema model, oxidative stress which responsible for inflammation associated with reactive oxygen species (ROS), exceeding the capacity of the endogenous antioxidant system [52]. From the obtained results we suggested that bioactive components in (OFI) MAE extracts protects cells from toxins such as free radicals through maintaining and increasing GSH and SOD levels. In addition to the decreased MDA, the main final gent of lipid peroxidation which gathers at the site of inflammation [53] and nitric oxide (NO) levels also proposed that these extracts might mediate the ROS-relevant lipid peroxidation that leads to a significance decrease of lipids damage in cell membranes. In agreement with our results, the extract of (OFI) flower has reported to reduce the lipid peroxidation products (MDA) of inflammation processes which are related to NO production [54].

In consistent with our findings, some studies have demonstrated that the ability of some flavonoids to inhibit cytokine production, may contribute to their anti-inflammatory properties [55] [56]. In addition to, ameliorate inflammation by of HO-1 mRNA expression upregulation with simultaneous reduction in the cytokine, TNF-α release [57]. Proinflammatory cytokines activate NF-κB which regulates several genes important in immunity and inflammation. It is well known that endothelial adhesion molecule expression is regulated by several redox-regulated transcription factors, including the master minder NF-κB [58]. A study reported that the anti-inflammatory activity of OFI, both *in vitro* and *in vivo*, inhibited the adhesion molecule overexpression and NF-κB transcriptional activity [59]. Moreover, IL-6 induces inflammatory responses in the cells via activation of the transcription factor STAT3 [60]. Other transcriptions factors such as NF-κB and TGF-β1 seems to be involved in transcription regulation of IL-6. Previous studies suggested several mechanisms for the anti-inflammatory effects of plant extracts containing phenolics [61].

The reduction of edema size is presented as a good indicator to determine the anti-inflammatory activity for different extracts. To confirm the obtained results from the edema size inhibition, pro inflammatory markers and cytokines were evaluated in rat serum samples. COX-2 is an inducible enzyme and is only expressed after an inflammatory stimulus [62]. The role of COX-2 is to synthesize prostaglandins for the induction of inflammation [63]. COX-2 is an important marker associated with anti-inflammatory mechanism and inhibition of edema. This edema model includes the release and the synthesis of inflammatory mediators at the site of inflammation such as prostaglandins for the second phase of inflammation (3–4 h after CARR injection) [64]. Previous study suggested that some flavones and flavonols may act as preferential suppressors of COX-2 [65] [66], while working on anti-inflammatory activity and the inhibition of arachidonic acid metabolism by flavonoids, revealed that flavonoid inhibit the arachidonic acid metabolism through the enzyme pathway which responsible for anti-inflammatory properties of these compounds.

4. Conclusion

This work demonstrates the possibility of exploiting microwave (MAE) and ultrasound (UAE) techniques to obtain *Opuntia ficus-indica* extracts remarkably enriched in valuable antioxidants and anti-inflammatory forms. The most essential reveals from this study are as follows:

- The optimum condition were found as extraction time of 5 min, 800 W and 15 min, 400 W for peel and pulp extracted using microwave assisted extraction (MAE), respectively.
- Avery high increase of total phenolic and flavonoids values were found in peel and pulp extracted using microwave assisted extraction by 74.1%, 115.3% and 55.4%, 105.8% respectively compared to conventional maceration.
- A high correlation between total phenolic and flavonoids contents for peel and pulp extracted using microwave assisted extraction and their *in vivo* biological activities were observed.
- The peel extracted using microwave assisted extraction with optimum condition at 5 min and 800 W reported the best antioxidant and anti-inflammatory activity.
- Overall, considering the results obtained, these advanced laboratory extraction methods could advantageously be scaled to a commercial plant within the framework of Green Chemistry.
- It is essential to provide new studies to isolate and identify the main constituent and its mechanism of action responsible for the anti-inflammatory activity.

5. Material And Methods

5.1 | Chemical materials and Reagents

The chemicals and reagents used in the study such as gallic acid (3, 4, 5-trihydrobenzoic acid), rutin, methanol (MeOH), Folin-Ciocalteu phenol reagent, aluminum chloride (AlCl3), and sodium bicarbonate (Na_2CO_3), were purchased from Merck (Darmstadt, Germany). The stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH), carrageenan and Indomethacin were purchased from Sigma-Aldrich (Darmstadt, Germany). Also, COX-2, IL-6, TNF-α, TGF-β1, NO, MDA, GSH, SOD concentrations were measured using ELISA kits purchased from (Wuhan Fine Biotech Co., China) and spectrometric kits purchased from Sigma-Aldrich (Darmstadt, Germany).

5.2 | Cell culture

The murine hepatoma cell line Hepa-1c1c7 was maintained as monolayer culture in α- modified Minimum Essential Medium Eagle (α-MEME) supplemented with 10% (v/v) heat-and charcoal–inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin sulfate in humidified incubator (Sartorius CMAT, Germany, 5% CO_2/95% air). At about 80% confluence, cells were routinely sub-cultured with Trypsin EDTA solution.

5.3 | Plant collection and identification

Opuntia ficus-indica fruits from locally termed as the "wild" cultivar were obtained from Abo-Hammad, Sharkiya, Egypt, during the harvesting season. The monthly climatological summary for August 2019, during which fruits had the optimum maturity, as a representative sample comprised of 500 fruits were used in the study. The taxonomic identity of the plant was confirmed with Prof. Dr. Ibrahim Ahmed El Garf, Department of Botany, Cairo University.

5.4 | Plant preparation

Randomly chosen fruits in the ripe stage of yellow to green color were washed with running water to remove glochids and impurities, air-dried and carefully hand-peeled. The peels (with a thickness of about 3-4mm) were separated from fruit pulp then chopped into small pieces and stored at -18°c in deep freezer.

The fruit juice was extracted using a blender (Moulinex, LM30214A, France). The obtained juice was filtered using a sterile cheese cloth to separate the seeds from the pulp. Then, the juice and the peel were lyophilized in freeze dry system to obtain dried powder of each one of them.

5.5 | Plant extractions

5.5.1| Conventional extraction methods

5.5.1.1| Maceration

The extraction was performed at room temperature by mixing 2.5 g of powdered (OFI) different parts (pulp and peel) with 25 mL 80% aqueous methanol in a sealed conical flask. Conventional maceration extraction was performed at different time intervals (15 and 30 min).

5.5.2| Innovative extraction methods

5.5.2.1| Ultrasonic assisted extraction (UAE) (20 kHz Probe).

A 20 kHz probe (Ultrasonic Processor UP400S (400 watts, 24 kHz, Hielscher, Germany) was employed for direct sonication extraction [67]. The horn tip was kept 1 cm below the solvent extraction surface inside the extraction vessel. Extractions of 2.5 g of OFI different parts (pulp and peel) using 25 mL 80% aqueous methanol were performed at different time intervals, 15 and 30 mins.

5.5.2.2| Microwave assisted extraction (MAE)

This method was performed using opened system microwave apparatus (MARS 240v/ 50Hz), 2.5 g of OFI different parts (pulp and peel) were extracted using 25 mL 80% aqueous methanol at different time intervals (for 5, 15 and 30 mins). Extractions were performed at two different powers (400 and 800 W).The temperature of extraction using MAE was fixed at 65–70°C considering the boiling point of methanol 64.7°C. [68].

5.6 | Determination of total phenolic acids or phenols (TPC)

Total phenolic content was determined by Folin–Ciocalteu according to the method described by [69] with some modification. Briefly, 20 µl of the sample was mixed with 100 µl of Folin–Ciocalteu reagent (diluted 1:10 with deionized water). After 15 min incubation, the mixture was neutralized with 80 µl of saturated Na_2CO_3. The absorbance of the mixture was measured at 760 nm after 2 h of incubation. Gallic acid was used as a standard for the calibration curve. Concentration of the total phenolics was determined from a standard calibration curve. The mean of three readings were used and the total phenolics were expressed as microgram of gallic acid (GAE) equivalent per 100 milligrams of the extract (µg /100 mg extract).

5.7 | Determination of total flavonoids (TFC)

The total flavonoids content was determined according to method as adopted by [70, 71]. Briefly, 40 µl of the sample were mixed with 40 µl of $AlCl_3$ (2% in methanol for HPLC grade) and the mixture was allowed to stand for 15 min. The absorbance was measured at 430 nm with a Zenyth 200rt microplate reader (Biochrom Ltd, Cambridge, UK) against appropriate blank. Rutin was used as a standard and the total flavonoids content was determined using the standard curve, the mean of three readings were recorded and the total flavonoids were expressed as microgram of rutin (RU) equivalent per 100 milligrams of the extract (µg /100 mg extract).

5.8 | Determination of *in-vitro* free radical scavenging (DPPH assay)

Various concentrations of (OFI) methanolic extracts (1ml) were mixed with 1 ml of methanolic solution containing DPPH radicals (6.10-5 mol/l). The assay method used in the present study was based on a modified procedure [72] which is based essentially on previously published literature [73].The mixture was shaken vigorously and left to stand for 30 min at room temperature in the dark. Absorbance was read using a microplate reader (tristar LP2, Berthol, Germany) at 520 nm. Rutin was used as the reference compound. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation:

$$\%RSA = [(A0 - A1)/A0] \times 100$$

Where A0 is the absorbance of the control (blank, without extract), and A1 is the absorbance in the presence of the extract. The extract concentration providing 50% of radical scavenging activity (EC50) was calculated from the graph of RSA percentage against extract concentration.

5.9 | *In vitro* anti-inflammatory study

RAW 264.7 Cells (500000 cell/ml) were seeded onto 96-well plates for 24 h. Cells were treated with either 0.1% v/v DMSO (negative control lipopolysaccharide {LPS}), 100ng/ml LPS+) or LPS in the presence of 6.26–100µg/ml of the extracts. Griess assay (32) as performed to determine N in triplicates of culture supernatants following 24 h exposure time in all groups. Briefly 100µL of culture supernatant from every well were mixed with equal volume of Griess reagent, mixed at room temperature an absorbance was

measured at 540 nm on a Tristarlb 942® microplate reader (Berthold, Germany). Inhibition (%) as calculate relative to the LPS only group (LPS+) [74]

5.10 | *In vitro* chemopreventive study

5.10.1 | NQO1 assessment

The induction of NQO1 in Hepa-1C1C7 cells was evaluated. Briefly, cells (3×10^5 cells/ml) were seeded onto 6-well plates and left for 24 h to adhere and form semi-confluent monolayers. Monolayers were treated with either vehicle (final concentration 0.1% v/v DMSO), peel MW 800 extracts (final concentrations of 100, 25 and 6.25 µg/ml) for additional 24 h [75]. In parallel, 4-bromoflavone (4-BF) was used as positive control for NQO1 induction. Monolayers were washed with ice-cold Dulbecco's PBS (1 ml/well). Cells were then scrapped in ice-cold homogenization buffer (25 mM Tris-HCl, pH 7.4, 250 mM sucrose and 5 µM FAD). Cell suspensions were then sonicated on ice for 5 s (20% amplitude). Sonicates were then centrifuged (15,000 ×g for 10 min) and the supernatants (cytosolic fractions) were aliquoted and stored at − 80°C freezer until tested for protein expression.

5.10.2 | Western blot analysis of (NQO1)

Hepa-1C1C7 cells were cultured and treated as mentioned above. NQO1 protein expression was assessed in cell sonicates by Western blotting as previously described with some modifications [75]. At the end of exposure, samples including vehicle control, positive controls (4-BF) and peel (MAE) at 800 W sample increasing concentration (6.25, 25, 100 µg/ml total proteins/lane) were resolved under denaturing conditions by electrophoresis (SDS-PAGE) on 10% acrylamide/bisacrylamide gel (200 Volts for 1 h). Resolved proteins were then transferred to nitrocellulose membrane at 100 V for 60 min. Membranes were blocked in 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h at 25°C and then probed overnight (4°C) with primary antibodies against NQO1 and β-actin (Abcam, UK). After three washes in TBST (5 min each), membranes were probed with 1:10000 dilutions of appropriate secondary antibodies (Abcam, UK) for 1 h at 25°C, washed three times in TBST and then developed using enzyme chemiluminescence (ECL, Pierce, USA, Invitrogen, USA), and bands were detected using CCD camera (UVP, UK).

5.11 | Toxicity studies of different Opuntia ficus-indica **extractions**

5.11.1 | In vivo **toxicological studies**

Swiss albino mice were housed in groups in stainless steel cages and kept under standard laboratory conditions. They were drinking water ad libitum and given pelleted food. The mice were acclimatized to the laboratory conditions for at least five days before initiation of the experiments.

5.11.2 | Toxicity and determination of median lethal dose (LD50)

Acute oral toxicity test was performed as per OECD guidelines[76]. Mice weighing 22–29 gm were randomly classified into four treated groups. LD50 of the promising extracts were assessed using the

method described by [77]. All groups were orally administered 1, 2, 3, 4, 5, 6 and 7 g/kg body weight of four (OFI) extracts, respectively. The LD50 of each extract was calculated according to the formula:

LD50 = Dm-Σ (Zxd)/n

Where, Dm is the minimum dose which kills all animals in the group, Z is the mean of dead animals in two successive groups, d is the constant factor between two successive groups, n is the number of animals of each group; and Σ is the sum of (Zxd).

The animals were observed continuously for first 24 hours and after 14 days for any signs of behavioral changes, mortality and body weight.

5.12 | Determination of anti-inflammatory activity (Experimental design)

The anti-inflammatory activities of the extracts were evaluated *in vivo* using carrageenan-induced rat paw edema model [78] [13] with some modification. Forty two adult male Sprague–Dawley rats weighing 160–190 g will be classified into 7 Groups (n = 6) as follows:

- **G1** served as the healthy negative control group (NCT) and received saline solution.
- **G2** served as the positive control group (PCT) induced with carrageenan (CARR) only, 0.1 ml of 1% freshly prepared solution of carrageenan in saline solution[57] in the sub-plantar region of left hind paw.
- **G3** induced with carrageenan and received (indomethacin; 10 mg/kg, p.o) as a reference drug [79] half an hour after carrageenan challenge
- **G4** induced with carrageenan and were orally dosed with 1/10 of LD_{50} dose of (OFI) peel extracted using conventional method (peel conv. extract) half an hour after carrageenan challenge.
- **G5** induced with carrageenan and were orally dosed with 1/10 of LD_{50} dose of (OFI) peel extracted by using MAE technique (peel MAE extract) half an hour after carrageenan challenge.
- **G6** induced with carrageenan and were orally dosed with 1/10 of LD_{50} dose of (OFI) pulp extracted by using conventional method (pulp conv. extract) half an hour after carrageenan challenge.
- **G7** induced with carrageenan and were orally dosed with 1/10 of LD_{50} dose of (OFI) pulp extracted by using MAE technique (pulp MAE extract) half an hour after carrageenan challenge.

5.12.1 | Calculation of rat paw edema inhibition %

Rat paw edema was assessed by using Vernier Caliper (SMEC, Shanghai, China) after carrageenan injection at 4, 3, 2, 1 and zero h. Difference in paw volume, assessed after injection of carrageenan

indicated the severity of edema. The inflammation inhibition percentage was assessed for each animal compared to controls and calculated by the following formula:

%I = 1- (dt\dc) ×100

Where ‘dt’ is the difference in paw volume in the treated group and “dc” the difference in paw volume in control group. “I” stands for inhibition.

5.12.2| Estimation of anti-inflammatory markers (COX-2, IL-6, TNF-α, and TGF-β1) in serum samples

This kit was relied on sandwich enzyme-linked immune-sorbent assay technology. Determination of IL-6 levels were performed using ELISA assay Kit. The change in color is measured spectrophotometrically at a wavelength of 450nm [80]. These kits were based on Competitive-ELISA detection procedure. The provided microtiter plate has been pre-coated with target. Through the reaction, target in the sample or standard competes with a fixed amount of target on the solid phase supporter for sites on the Biotinylated Detection Antibody specific to target. Excess conjugate and unbound sample or standard were washed from the plate, and Streptavidin-Biotin Complex (SABC) was added to each microplate well and incubated. Then 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate solution is added to each well. The enzyme-substrate reaction is terminated and the color change is measured spectrophotometrically at a wavelength of 450nm. The concentration of target in the samples is then determined by comparing the OD of the samples to the standard curve.

5.12.3| Estimation of antioxidant parameters in serum samples

5.12.3.1| Nitric oxide measurement

Nitric oxide (NO) was rapidly oxidized to nitrite and nitrate which were utilized to quantitate NO production. NO assay colorimetric kit was used to measure the total nitrate/nitrite in a simple two-step process. Firstly, nitrate reductase was used to converts nitrate to nitrite. Then, Griess reagent was used to converts nitrite to a deep purple azo complex. The amount of the azochromophore accurately revealed nitric oxide quantity in the samples. Finally, optical density was measured at A540 nm by the microplate reader (BMG Labtech, Germany) [81].

5.12.3.2| Determination of lipid peroxidation levels; Malondialdehyde (MDA)

MDA levels, as a lipid peroxidation product, were assessed using ELISA assay Kit (Wuhan Fine Biotech Co., China). In this method, the thiobarbituric acid (TBA) reactive substances (TBARS) quantity was detected as a MDA production index in serum samples based on nmol per ml of serum. The change in color is measured spectrophotometrically at a wavelength of 450nm [82].

5.12.3.3| Determination of Superoxide Dismutase (SOD)

Determination of SOD enzyme activity was performed using ELISA assay Kit (Wuhan Fine Biotech Co., China). In brief, after reduction of 2-(4-iodophenol)-3-(4-nitrophenol)-5-phenyltetrazolium chloride by

xanthine oxidase, a red formazan product was produced. This reduction was suppressed by SOD and produce colored complex. Its absorbance was quantified at 450nm [82].

5.12.3.4| Determination of Reduced glutathion (GSH)

It was determined through colorimetric method using 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) according to protocol provided by the kit. Briefly, DTNB reacted with reduced form of thiol (–SH) groups and makes complex. The absorbance was read at 450 nm to estimate the GSH levels [83].

5.13 | Statistical analysis

Statistical analysis was done using the SPSS (Statistical Package for the Social Sciences) version 20.0 statistics software (IBM Corp., Armonk, NY, USA, 2011). The mean (± standard deviation) values of bioactivity parameter analyses were evaluated using the Microsoft Excel spread sheets. The t-test was applied at the confidence level of $p < 0.05$ for significance and $p < 0.01$ for high significance when comparing between groups.

Declarations

Ethics approval and consent to participate

All animals were retained as per the approvals of the guiding principle in the care and use of laboratory animals permitted by *Medical Research Ethics Committee*, National Research Centre, Cairo, Egypt, ethical approval number (20-048).

Consent for publication

All co-authors consent to submission of this work for publication.

Competing interests

The authors declared that they have no competing interests.

Availability of data and materials

All data are available as figures and supporting figures.

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Authors' contributions

I.S., M.V., I.C. and T.M. designed the plan of work; K.S., N.A. and A.A.; contributed to development and design of methodology; I.S., A.H., E.B., K.M. conducted research, performed the experiments; E.B., A.F.,

M.A. contributed to data collection, investigation and interpretation of the results; I.S. contributed to writing and drafting the article; A.F., T.M. provided critical revision of the article; All authors discussed the results and contributed to the final manuscript.

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Figures

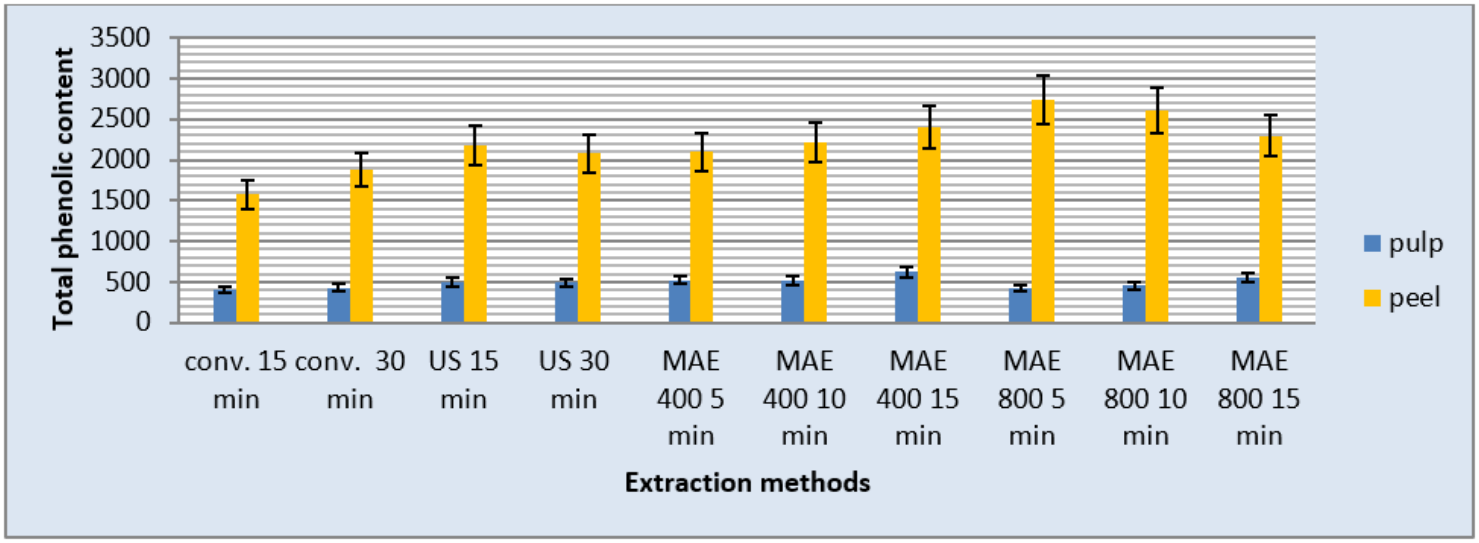

Figure 1

Total phenolic content for different (OFI) extracts

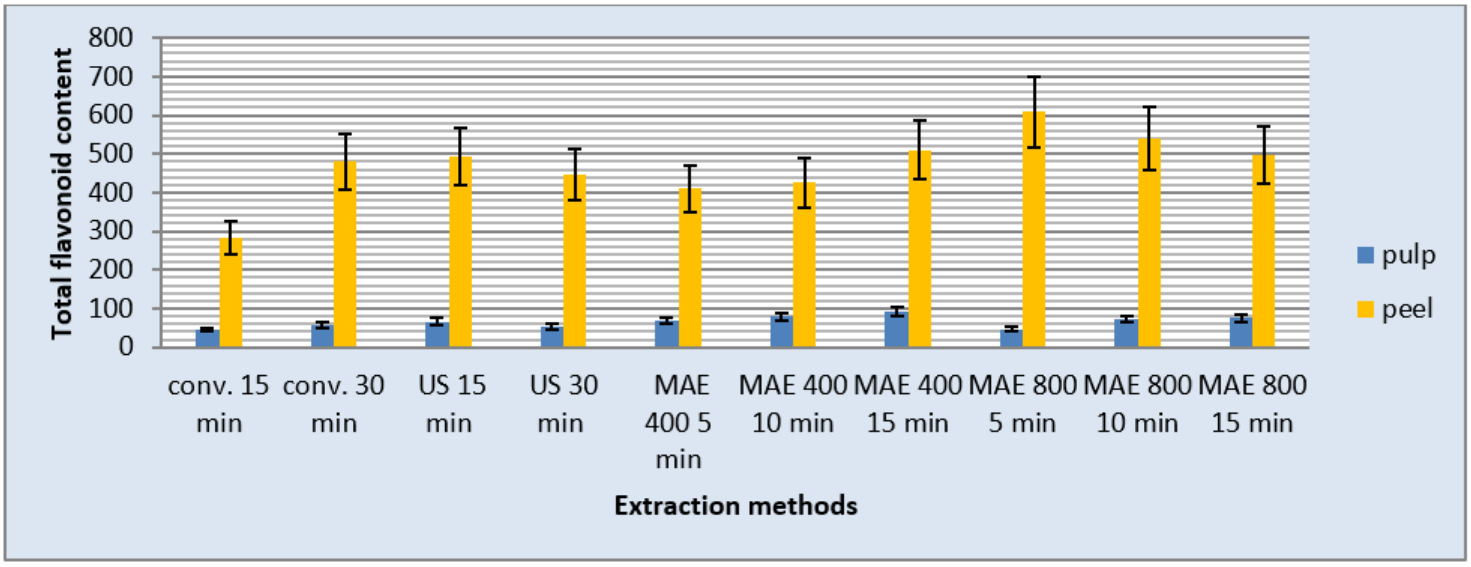

Figure 2

Total flavonoid content for different (OFI) extracts.

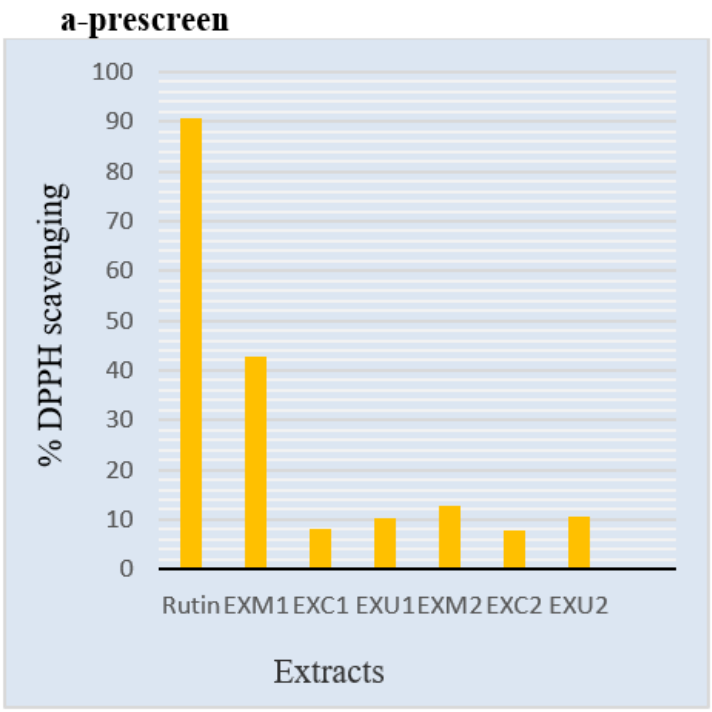

Figure 3

Preliminary screening of (OFI) different parts extracts obtained using conv., MAE, and UAE methods compared to rutin (0.36 µg/ml) as the reference radical scavengers.

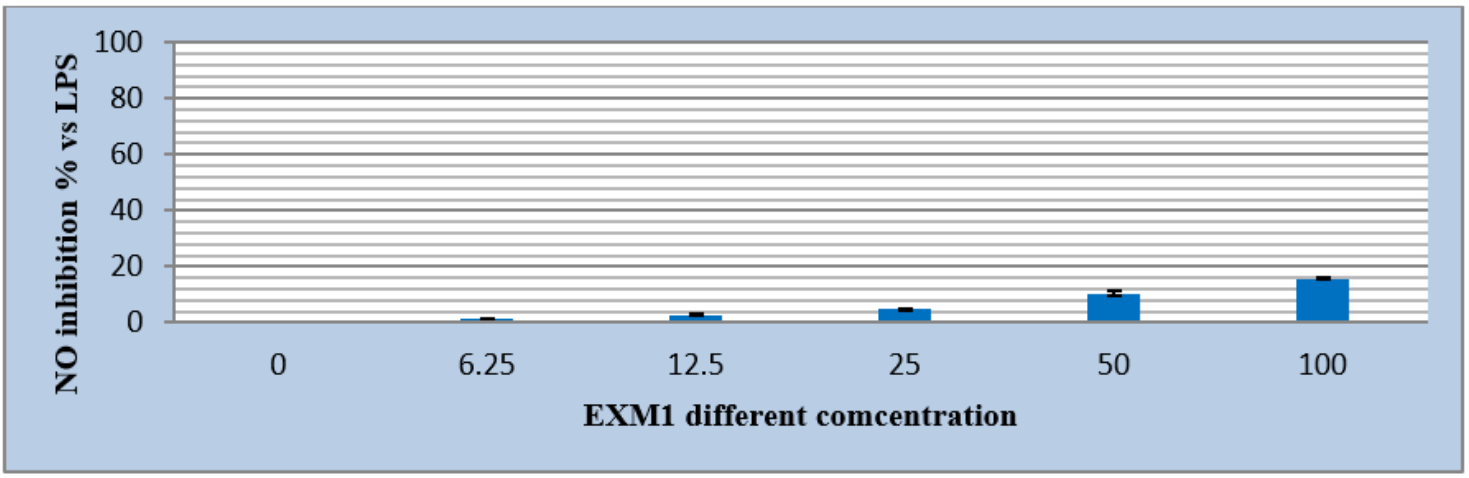

Figure 4

Determination of the (NO) inhibition % for EXM1 at different concentration.

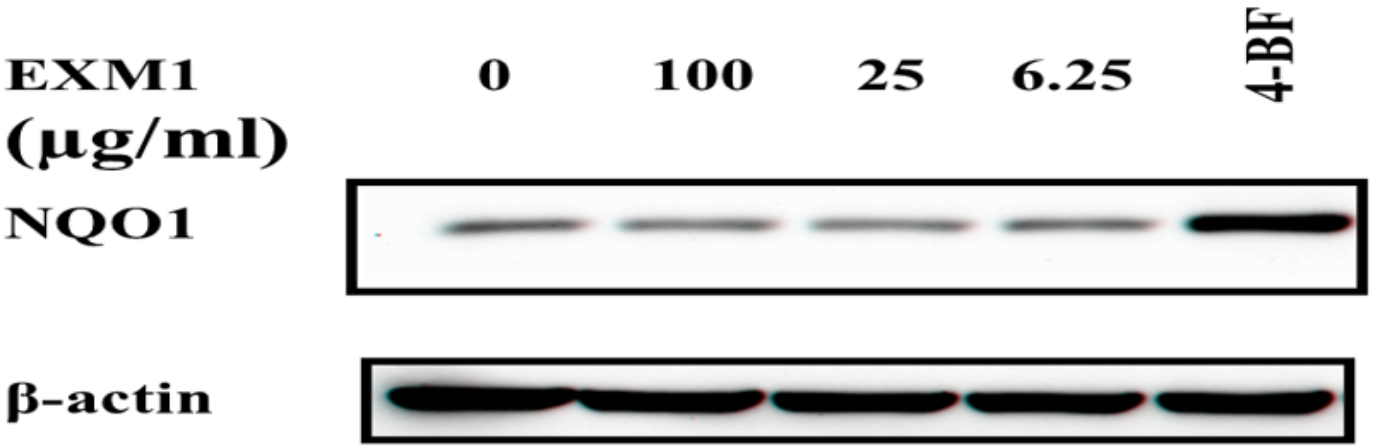

Figure 5

Assessment of the cancer chemopreventive potential by Western blot analysis of the NQO1 protein expression for EXM1 at different concentration *in vitro*.

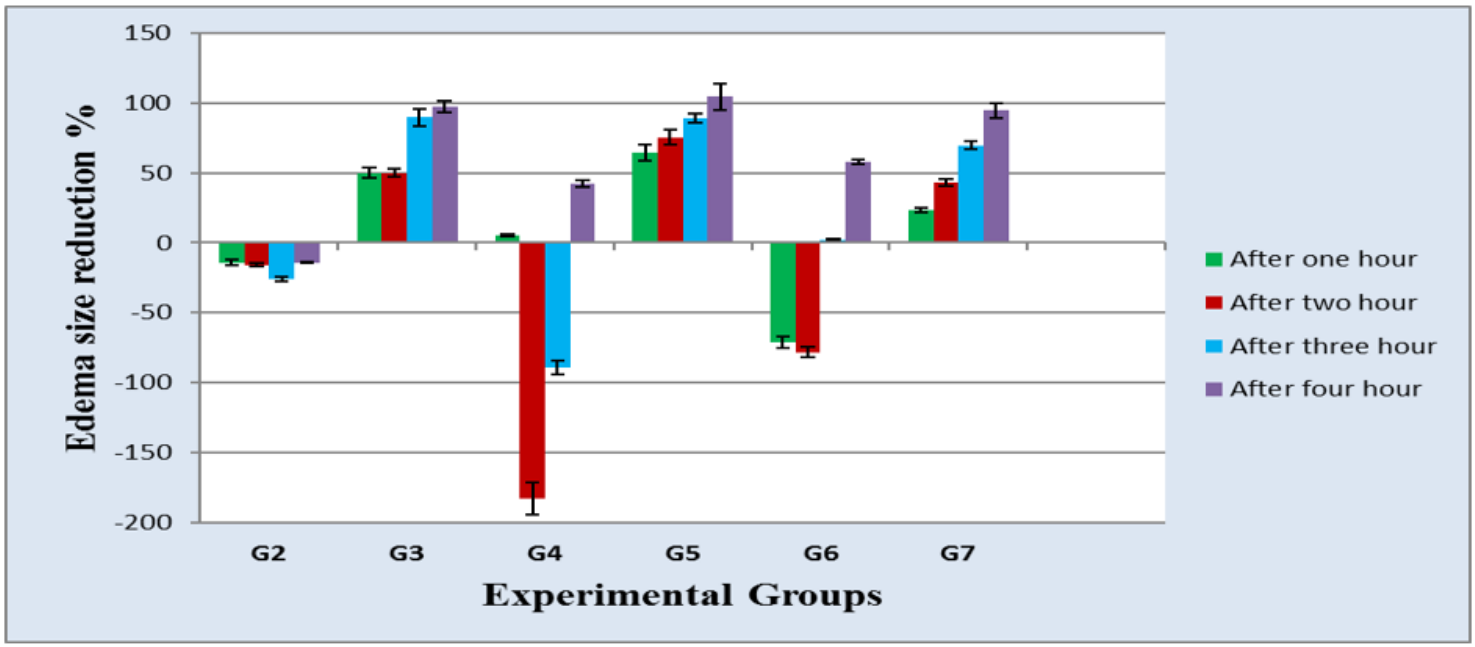

Figure 6

Rat paw edema curve of different treated groups where G2: treated with carrageenan only (PCT), G3: treated with indomethacin (NCT), G4: treated with EXC1 extract, G5: treated with EXM1 extract, G6: treated with EXC2 extract and G7: treated with EXM2.

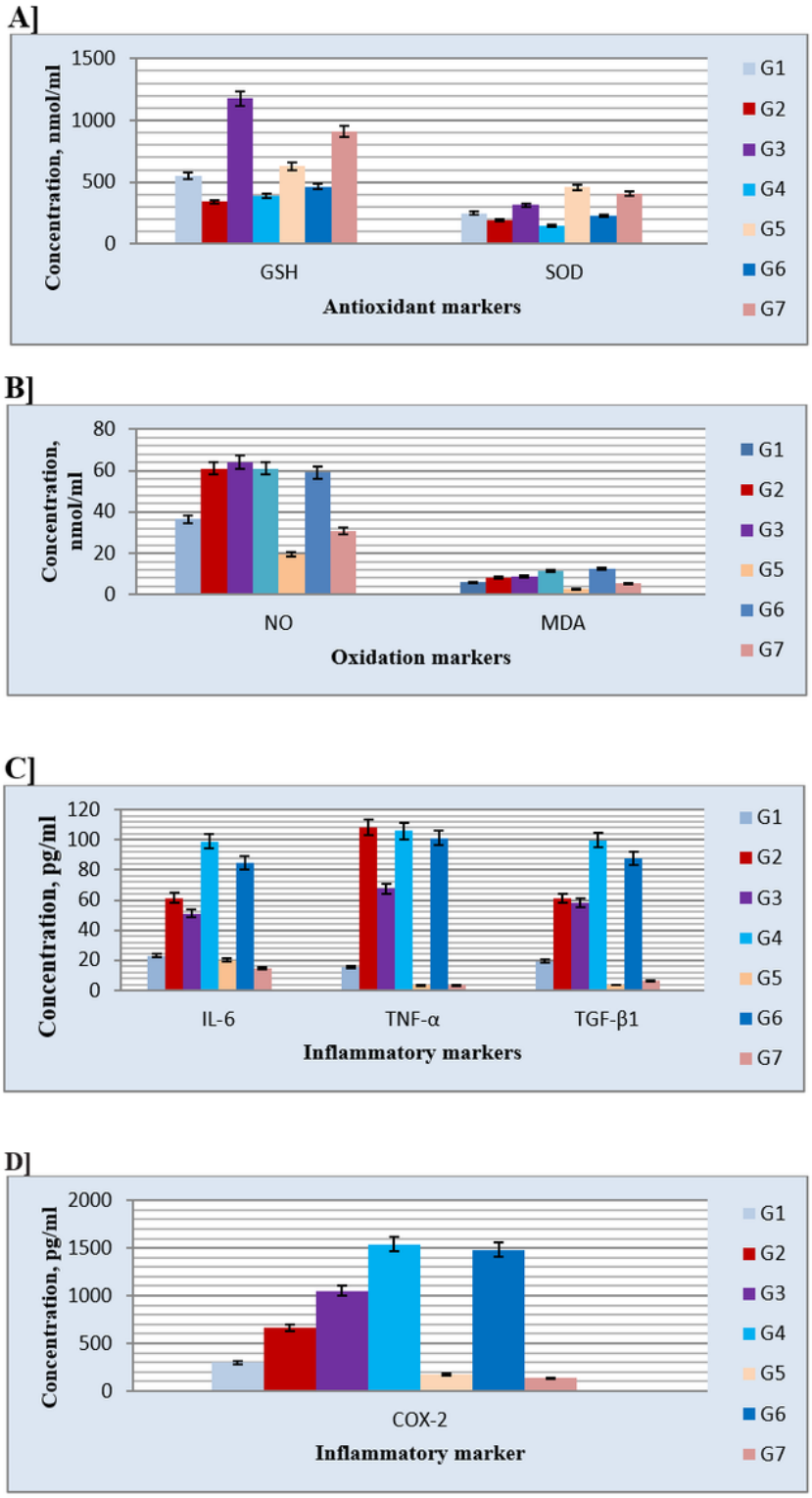

Figure 7

Determination of GSH, SOD (A), NO, MDA (B), IL-6, TNF-α, and TGF-β1 (C), COX-2 (D) in serum samples of different treated groups where G1: negative control (NCT) ,G2 :treated with carrageenan only (PCT), G3: treated with indomethacin after carrageenan challenge ,G4: treated with EXC1 extract ,G5: treated with EXM1 extract, G6: treated with EXC2 extract and G7: treated with EXM2.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Fig5.docx