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Original Article

Anti-Inflammatory Activity of Root Ethanolic Extract of Harpagophytum procumbens on Human Peripheral Blood Mononuclear Cells and Screening of its Bioactive Compounds by GC-MS Method

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Abstract:

Background: A traditional African medicine consisting of an anti-inflammatory fame is the Devil s Claw, e.g. Harpagophytum procumbens. Its extracts have a broad application in Europe on the instances of musculoskeletal pain and low back pain as the therapy of such inflammatory issues. It has been found out that Harpagophytum procumbens is capable of alleviating pain and reducing the need of NSAIDs and possibly as analgesic and an anti-inflammatory agent, mainly in osteoarthritis.

Procedures: The powder was resieved with certified sieve. The volatility and polyphenols are the classes of phytochemicals whose biocompatibility and solubility dictated the choice of the solvents. An inert GCMS selective detector 6890N/5973 of Agilent Technologies, USA, was used to analyze by GC-MS. PBMCs isolation and harvesting of peripheral blood samples. The pretreatment and incubation of PBMCs with the three different doses of Harpagophytum procumbens was done after which the incubation time was permitted to elapse after two hours of which LPS (1.0 0g/mL) was added to stimulate the cells. The concentration of the pro-inflammatory mediators was measured after removing and storing at 70 25C the supernatants of the LPS-stimulated and the pretreatment PBMCs after 24 hours.

Results: GC-MS analysis belongs to the number of the common techniques that could be applied to study the chemical composition of the plant extracts, the one of H. procumbens included. The method is detectable to volatile and non-volatile root and plant extract constituents. Over 31 compounds are discovered as a result of the research. Speaking about the most frequent kind of chemicals we may point out the existence of p-Vinylguaiacol, Decahydronaphthalene, 1-methylcyclohexane-1-carboxamide, Tetradecanoic acid, 1-methylethyl ester, 6-methoxypyridine-3-carboxylic acid, Pentadecanoic acid, 15-amino, Octahydrophenanthrene, Oleic acid, trimethylsilyl ester, Cell viability of PBMCs after incubation with six concentrations (0, 10, 20, 40, 80, 160, and 320 ug/ml) of Harpagophytum procumbens at different time points (24, 48 and 72 h) was found to be 94.83+/-3.1, 91.13+/-3.7, 90.03+/-3.8, 87.03+/- The production of pro-inflammatory mediators (TNF-alpha, Interleukin-1b and Interleukin-6 Pg/mL respectively) was recorded as (3.96, 8.27, 4.91, 3.52, 30.46, 15.70, 12.50 and 9.58) Pg/mL respectively in the effect of Harpagophytum procumbens (0, 10, 60, 150, LPS But Interleukin-1 5.50, 10.49, 3.67, 1.50, 35.70, 16.88, 10.00 and 6.49 Pg/mL was recorded respectively. During the same time the Interleukin-6 levels were (35.00, 60.71, 53.98, 18.93, 180.01, 176.61, 144.00 and 133.58) Pg/mL respectively.

Keywords: Anti-Inflammatory, Peripheral Blood Mononuclear Cells, Bioactive Compounds, GC-MS Technique

Introduction:

Inflammation is a normal immune system defence process and it helps the body to deal with worn-out cells and particles, dangerous infections, and environmental toxins. It all starts with monocytes and macrophages that are two key players of such response [1, 2]. They secrete an entire host of chemicals that are connected with inflammation. Mediators can be cytokines, chemokines, enzymes, peptides, complement system elements, amines and lipid molecules. The best pro-inflammatory cytokines in the body are tumour necrosis factor-alpha (TNF-alpha), IL-1 beta and IL-6, IL-8 and IL-11. Some of the members of the pro-inflammatory mediator category include the glutathione peroxidase prostaglandin E2 (PGE2), superoxide dismutase (SOD), NADPH oxidase (NOX), and inducible nitric oxide synthase (iNOS), (GPx), cyclooxygenase-2 (COX-2). Devil claw (Harpagophytum procumbens DC) is a member of the Pedaliaceae family and also called by several other names, which include grappling plant, wood spider and harpago [3-5]. People have used it since time immemorial to heal pain, fever, malaria and indigestion. Also, both animal and human experiments have suggested the utility of H. procumbens extracts in rheumatic illnesses. In making sure that the quality of herbal drugs one should adequately identify the crude herbal material and it has been discovered that H. procumbens extracts aide in curing acute or sub-chronic inflammation in rats. Meanwhile, GC-MS could be regarded as the most suitable technique to investigate the chemical composition and standardisation of MPRM [6, 7]. Rapid separation of a large number of volatile chemicals is accomplished by the high selectivity of capillary columns. GC-MS is widely used in studies encompassing herbal products because of the performance of the method in separating and identifying substances [8, 9]. The research had two goals to identify the chemicals in the roots of H. procumbens and their effect on the human peripheral blood mononuclear cells.

Materials and Methodsm

The strategies of collecting plants and extracts of them

It is made of a dried root of H. procumbens that is extracted. The process was started with 300 g of the finely chopped roots which were repeatedly cleaned in 99 percent ethanol (v/v). The slices were rinsed and ground in electric miller within 10 minutes. The obtained powder was sifted through a sieve with 4 mm openings. After stirring in the dark at 58 o C during 24 hours, the mixture was dissolved in 1:5 ethanol and water. This was filtered using a 0.45 mum membrane by filtration. after collection, the fraction was dried at 80 ◦C in a static dryer with 5% maltodextrin carrier. The powder was also tested with the help of a certified sieve that was 200 -um in diameter, 100 meshes, and 0.150 -um net light. Before it was used, it was kept at room temperature, in a dry and dark place. These solvents were chosen because of being bio compatible and because they are able to dissolve a large variety of phytochemicals which are largely volatile compounds and polyphenols. The extract was dried and kept in an airtight and dark bottle. It was kept at 20 ◦C until it was evaluated. This was followed by dissolving the extract in distilled water. The latter is followed by reconstitution of the dried extract in distilled water. In both experiments, the work was done with the following dilutions of extract: 10, 20, 40, 80, 160 and 360 Michael off/mL. All the materials used in the immunopharmacological studies whether synthetic, biologic or natural compounds were free of endotoxins.

Analysis Gas Chromatography – Mass Spectroscopy.

The instrument (an abbreviation, meaning gas chromatograph/mass selective detector) that was used to carry out the experiment was made by Agilent Technologies of the USA. The column constructed using HP-5MS had dimensions 30 m 0.25 mm and the layer thickness was 0.25 mm. Helium gas was of 99.999 percent purity and 0.5 millilitres of the gas was pumped into the injector at the rate of 1 millilitre per minute. Internal temperature of evaporator detector was 300 C. The injection was maintained at 250 o C and the ion-source at 280 o C. The oven was heated at 50 o C, 5 min, then 4 o C/min to 220 o C and finally, 10 o C/min to 300 o C. This run was finished after

10 min of isothermal heating at 300 C. Component identification Identification of components by interpretation of GC-MS mass spectra against the NIST database took about 50 min and requires consideration of more than 62 000 patterns. We analysed the concentration of the constituents of the final product as part of the standard operating procedure. In this experiment we have taken those parts that gave a probability greater than 90 percent just by chance. They were calculated separately three times each.

Selection of healthy controls

In the test, a mean of thirty six point four year old men participated. The volunteers were asked to stop using any type of immunosuppressant and herbal drugs, they were also supposed to lack any traces of inflammation and autoimmune disorders, bacterial or viral infections. The candidates were required to be non-smokers; they needed to lack hypertension or metabolic issues; they were also not allowed to consume corticosteroids, immunosuppressants, or herbal drugs. All of them were informed about the procedures of the study and a type of informed permission signed by them prior to their participation in the research or collection of their samples.

Peripheral blood sampling and isolation of the PBMCs

Five milliliters of blood of each individual was taken in vials with EDTA. The PBMCs separation was performed by density centrifugation immediately after collection. A sterile container was used in adding blood samples to a PBS one part solution and one part sample. Diluted blood (1:1) was centrifuged in an equal volume of cell density gradient medium (Lympholyte-H, Cedarlane, Ontario, Canada) at 2000 RPM (900xg), 30 minutes, at room temperature (18 -20 C) without hesitation. The white layer was thereafter removed and the PBMCs washed twice with cold PBS as they were centrifuged at 1200 RPM, 10 minutes at room temperature. The cell pellets were then resuspended in 1 millilitre ice-cold PBS. PBMCs number was then measured with the help of hemocytometer and their viability with the help of trypan blue dye test. The samples whose viability was more than 95 percent were re-analyzed and tested.

Options under control and research categories

The eight groups are as follows:

1. All the groups utilized unstimulated PBMCs. The PBMCs that were fully in RPMI-1640 controlled each group. The sample was diluted until the extracts of Harpagophytum procumbens contained 0 0g/mL.

2. Harpagophytum procumbens extracts were added into unstimulated PBMCs at a final concentration of 10 0g/mL.

3. PBMCs were exposed to Harpagophytum procumbens 30ug/mL extract.

4. PBMCs without pericytes that were non-stimulated and incubated with a Harpagophytum procumbens extract at a concentration of 90microg/mL.

5. LPS-activated PBMCs. In this well, the cells were subjected to 1.0 10 -6 g/mL of LPS concentration. The sample was diluted until the extracts of Harpagophytum procumbens contained 0 0g/mL.

Harpagophytum procumbens extracts were pre-incubated with PBMCs (10 ug/mL) Prior to incubation of the PBMCs with LPS (1.0 ug/mL).

7. PBMCs were incubated with 30 g/mL of Harpagophytum procumbens extracts and further incubated with 1.0 g/mL of LPS each at 2 hours.

8. PBMCs were incubated with Harpegophytum procumbens extract at a final concentration of 90 5g/mL which was pre-incubated before the addition of LPS at a final concentration of 1.0 5g/mL.

Cell culture

RBMI-1640 is a full culture medium that was supplemented with the PBMCs. It also supplemented the same medium with 10% foetal bovine serum (FBS), 2 mM L- glutamine, 100 IU/mL penicillin and 100 mg/mL streptomycin that were purchased from Gibco BRL Co. Ltd. in NY, USA.

Directing peripheral blood mononuclear cells

After pre-incubation of the PBMCs with the various dilutions of Harpagophytum procumbens (2 hours), LPS (1.0ug/ml) was then added to the cells. Following the addition of all the ingredients, the contents of the samples were swirled gently and then incubated at 37 ◦C and 95 percent humidified atmosphere (containing 5 percent CO2). The concentration of pro-inflammatory mediators was to be measured by collecting the supernatant of LPS-stimulated PBMCs after 24 h and storing it at 70 ◦C.

Cell viability assay

The effect of Harpagophytum procumbens on PBMCs was established through the MTT assay technique. This is the process employed when the colourless MTT is turned blue as a result of the formation of formazan in the existence of the enzymes referred to as dehydrogenases found in living cells. In our experiment of MTT, we have taken the same procedure as Mosmann had done earlier. JET BIOFIL 96-well flat-bottom culture plates were used to seed the PBMCs at a density of 1 x 10 1 PBMCs/well and incubated at 37 C, 95% humidity, and 5% CO 2. In the experiment the cells were divided into seven groups; one group which was treated with normal RPMI-1640 served as the control, and other six groups which were incubated with various concentrations of Harpagophytum procumbens (10, 20, 40, 80, 160 and 360) at 24, 48 and 72 hours respectively. The plate was thereafter incubated at 37 ◦C with MTT over four additional hours after which the supernatant in every well was discarded and 200 ml DMSO added to dissolve the newly developed formazan crystals. Viability of the untreated PBMCs was taken as a control and the experiments were carried out in triplicate. The colour was read at 570 nm absorbance in PerkinElmer Multimode Microplate Reader. The next step was the calculation of viability of PBMCs and its representation in percent.

Cell Viability (%) = [(Mean absorbance of treatment group/ Mean absorbance of group without treatment)] x 100.

ELISA Assay is an enzyme-linked immunosorbent Assay.

TNF- alpha, IL-6 and IL-1 Beta were detected in the culture supernatants of cells using ELISA kits according to the manufacturers instructions. The ELISA reader (SynergyTM HTX Multi-Mode Microplate Reader by BioTek Instruments, Inc.) was used to measure the optical densities of the samples at a light wavelength of 450 nm and the experiments were repeated thrice. The findings were attributed to the standards supplied by manufacturer of kits.

Analysis of Data Statistic

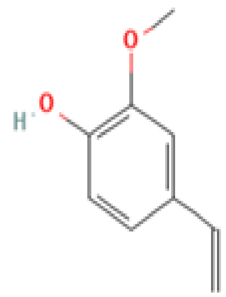
The SPSS Inc. in Chicago, Illinois, USA (version 20)designed the statistical tests which were carried out on the SPSS software installed. In order to enable us to compare the variables of the different groups, we performed an analysis of variance (ANOVA) and posthoc Scheffe test. $P<0.05$ was considered to indicate significance and the data is represented as the mean +/- SEM. When the results of the MTT assay were to be seen, a percentage was followed by a standard error of the mean (SEM).

Results and Discussion:

The result of GC/MS analysis indicated that the root of Harpagophytum procumbens contains fifteen chemicals. GC-MS is a viable technique of identifying the phytocompounds present in plant extracts and the structure adopted by them. In this method the chemicals are so well treated that there may be minimum possibilities of error and the fingerprint comes out correct. Besides this, GC-MS also gives information of quantitative data along with a mass spectral database that is highly helpful in relating the bioactive compounds with their medicinal action. Gas chromatography is usually used to isolate organic substances. In GC, a column is used in which the analyte is made to venture through the aid of a gaseous solvent called the carrier gas [13-15]. The various fragments in the sample can be seen and matched with the mass spectra in the databases to deduce the composition of the compound. With GC-FID, it is possible to separate mixtures of compounds and also identify the compound as pure samples of the same compounds are required. The method allows you to find out the amounts of chemical constituents that are important to you using the common analytical curve. The terms biologically active and bioactive are synonymous. Any bioactive compound has effect on the living systems. A bioactive compound is a compound that has direct impact on an organism. some bioactive compounds may have healthy results and impacts whilst others may have adverse outcomes and impacts depending on their properties, doses, and the way they are delivered into the body. Most researchers claim that the intake of the bioactive compounds in their adequate levels are capable of preventing and handling various diseases [16, 17]. The food sector is now aiming at producing functional foods so as to avert the occurrence of health complications such as cardiovascular diseases, obesity, diabetes and cancer since the incidences of individuals developing these health complications have risen nowadays. The experts have discovered that intake of the bioactive compounds can be of help as they contain functional foods. Acceptance of bioactive ingredients in food helps to improve the nutritional status of the food and provides health benefits to the brain, heart, and the immune system in addition to eliminating the possibilities of contracting chronic diseases. One of them is antioxidants, which are mainly antioxidants, which preserve the human cell and minimize the chance of chronic and cardiovascular diseases [18-21]. They are also called anticancer drugs. The other bioactive components are the omega-3, 6, and 9 fatty acids [22, 23]. These are referred to as polyunsaturated fatty acids since they are helpful to the immune system as well as heart disease and cancer prevention.

p-Vinylguaiacol

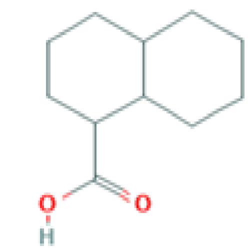
$C_9H_{10}O_2$



M.W.: 150.17 g/mol

Decahydronaphthalene-1-carboxylic acid

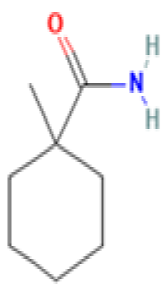
$C_{11}H_{18}O_2$



M.W.: 182.26 g/mol

1-methylcyclohexane-1-carboxamide

$C_8H_{15}NO$



M.W.: 141.21 g/mol

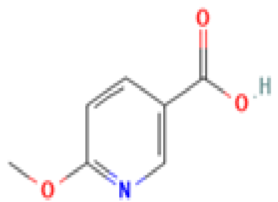
Tetradecanoic acid, 1-methylethyl ester

$C_{17}H_{34}O_2$

M.W.: 270.5 g/mol

6-methoxypyridine-3-carboxylic acid

$C_7H_7NO_3$



M.W.: 153.14 g/mol

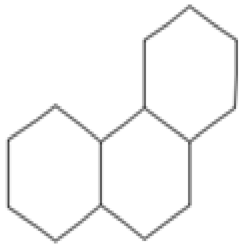
Pentadecanoic acid, 15-amino-

$C_{15}H_{31}NO_2$

M.W.: 257.41 g/mol

Octahydrophenanthrene

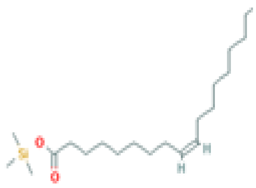
$C_{14}H_{24}$



M.W.: 192.34 g/mol

Oleic acid, trimethylsilyl ester

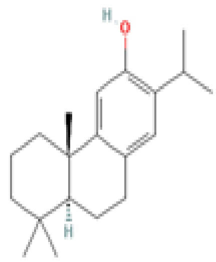
$C_{21}H_{42}O_2Si$



M.W.: 354.6 g/mol

trans-Ferruginol

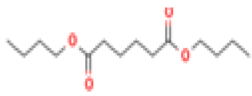
$C_{20}H_{30}O$



M.W.: 286.5 g/mol

Dibutyl hexanedioate

$C_{14}H_{26}O_4$



M.W.: 258.35 g/mol

Figure 1. Cell viability of PBMCs [MTT reduction assay] treated with different concentrations of Harpagophytum procumbens during (24h)

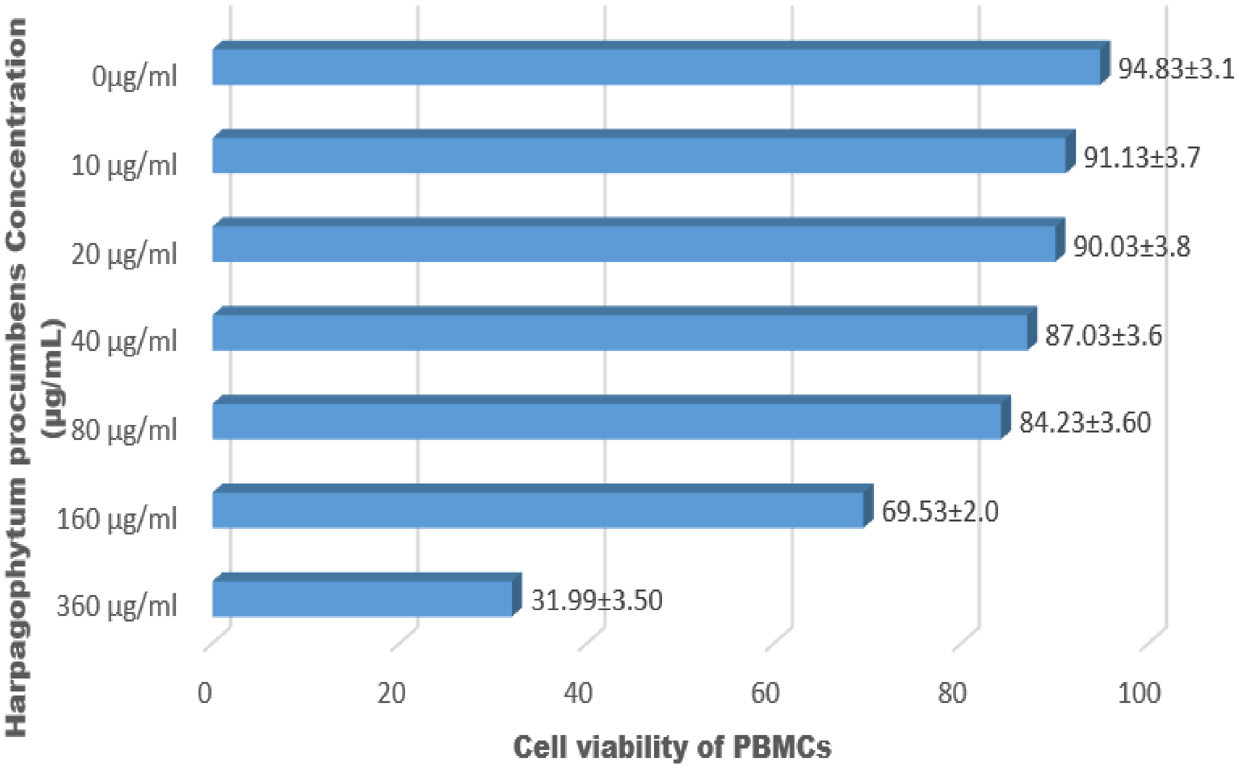


Figure 2. Cell viability of PBMCs [MTT reduction assay] treated with different concentrations of Harpagophytum procumbens during (48h)

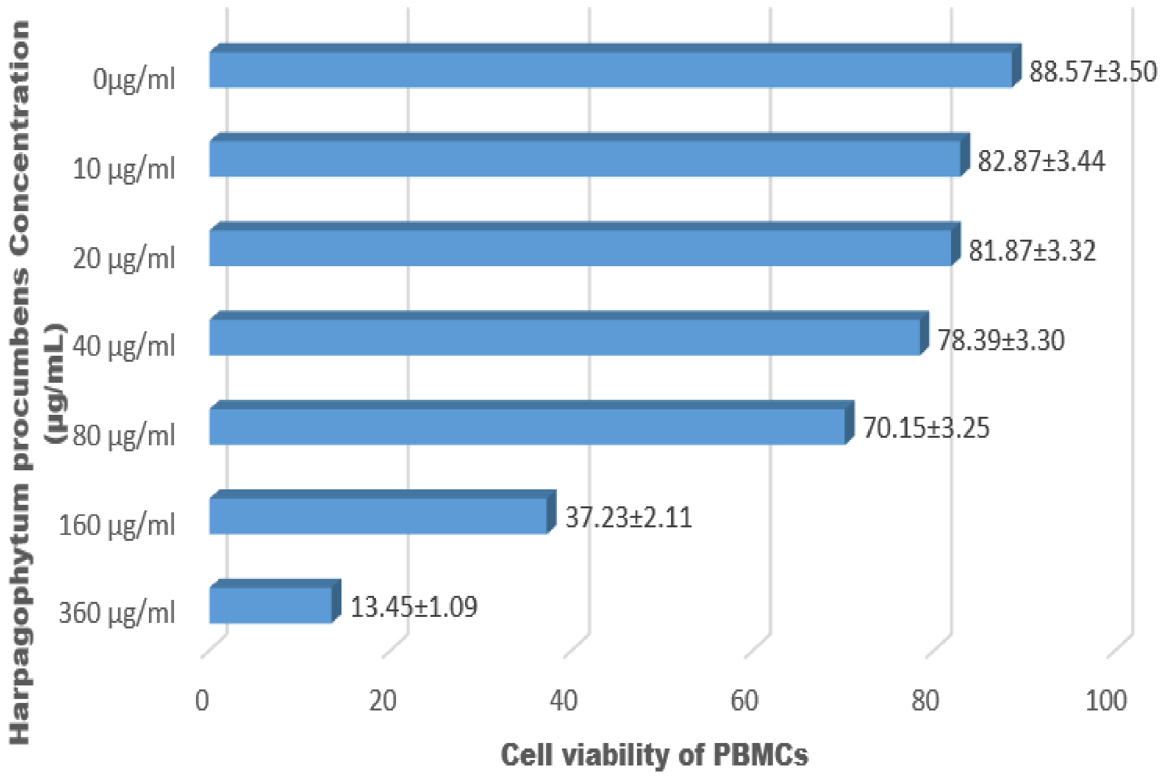


Figure 3. Cell viability of PBMCs [MTT reduction assay] treated with different concentrations of Harpagophytum procumbens during (72h)

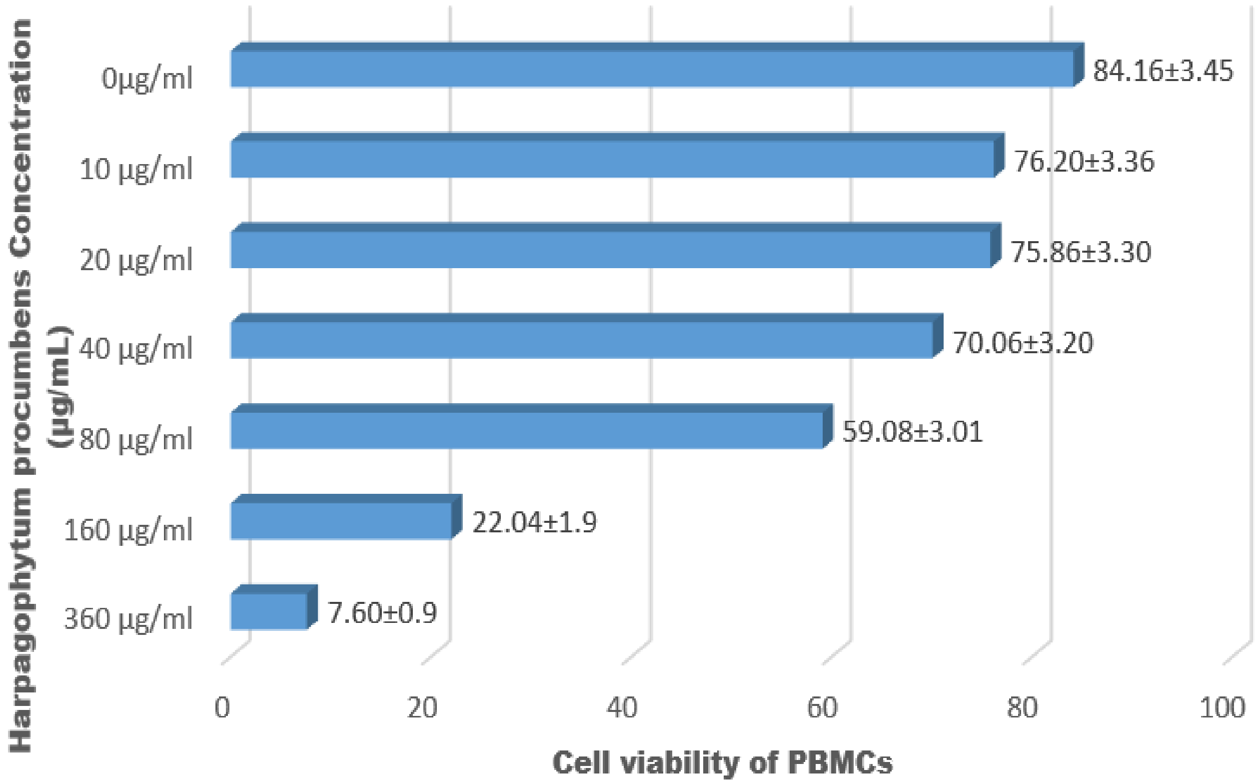


Figure 4. Effects of Harpagophytum procumbens on the production of pro-inflammatory mediators (TNF-α) Pg/mL

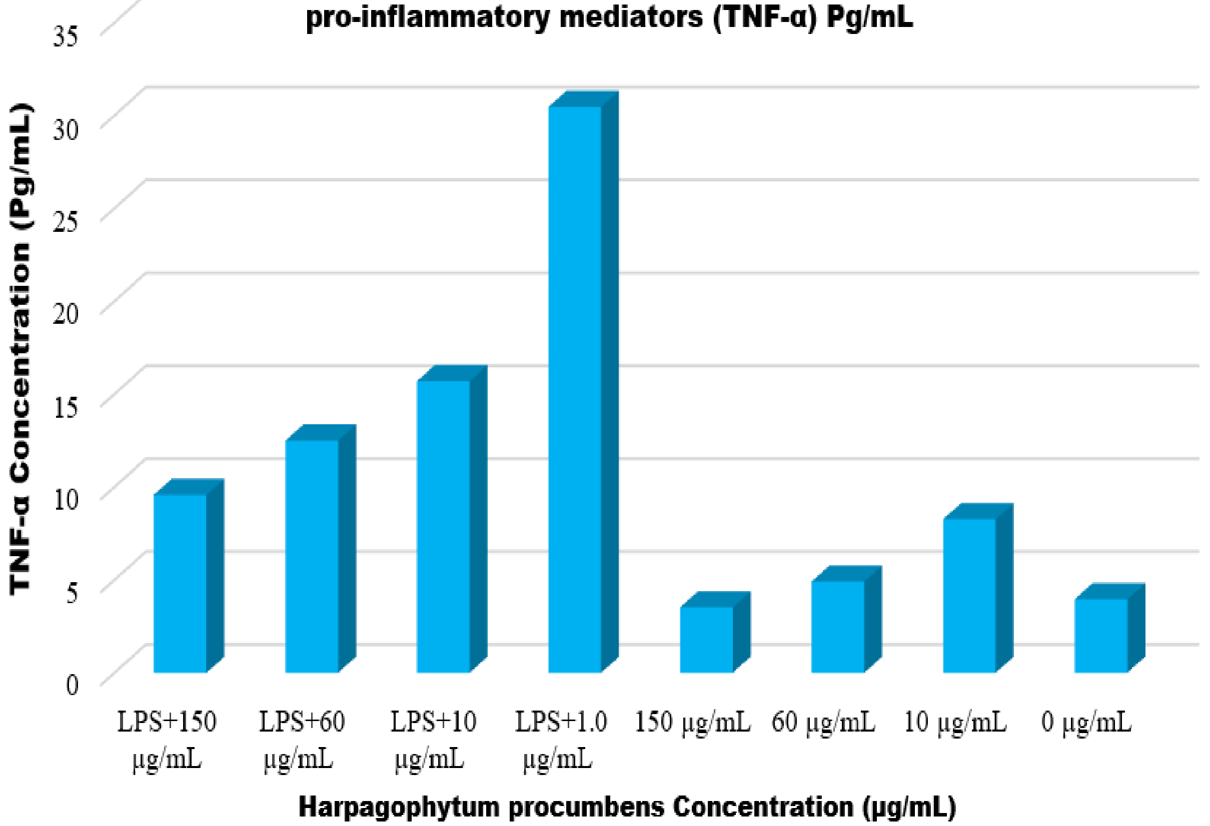


Figure 5. Effects of Harpagophytum procumbens on the production of pro-inflammatory mediators (Interleukin-1β) Pg/mL

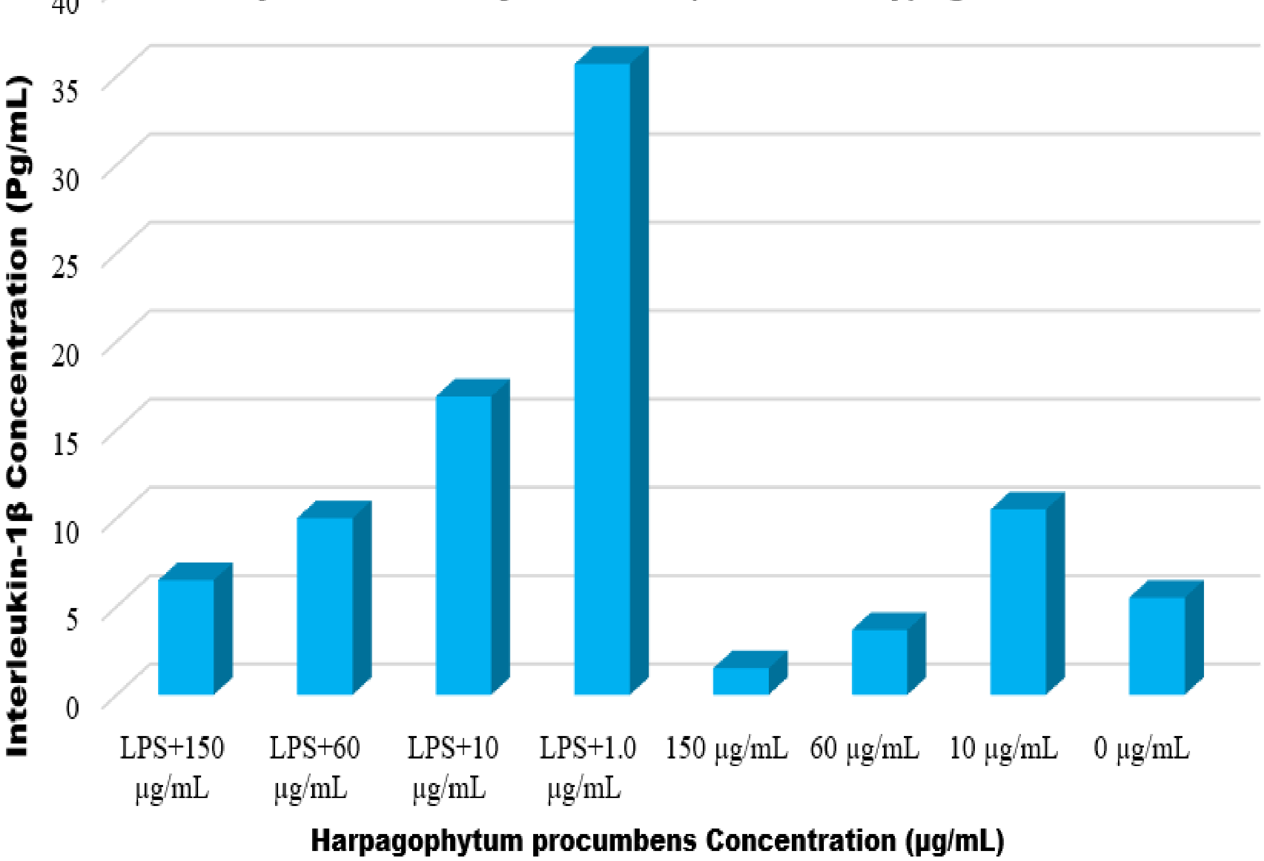
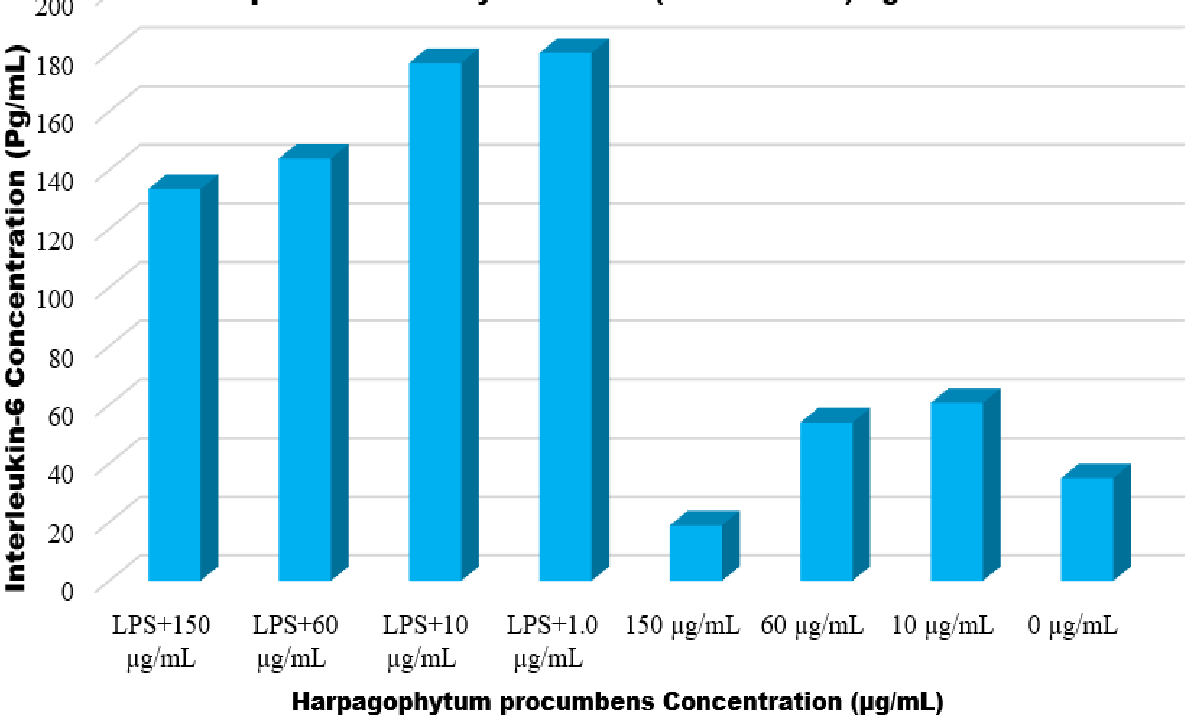


Figure 6. Effects of Harpagophytum procumbens on the production of pro-inflammatory mediators (Interleukin-6) Pg/mL



The outcome of the MTT reduction assay was represented in Figures (1-3) to denote the effect of Harpagophytum procumbens on cell viability. Percentage cell viability of peripheral blood mononuclear cells (PBMCs) following exposure to six concentrations of Harpagophytum procumbens at three time points (24, 48 and 72 hours). The results of Harpagophytum procumbens at six different concentrations and non-treated PBMCs (control) are as follows: (0, 10, 20, 40, 80, 160 and 320 ug/ml) recorded 94.83±3.1, 91.13±3.7, 90.03±3.8, 87.03±3.6, 84.23±3.60, 69.53 The percentages of cell survival in all the four exposures to Harpagophytum procumbens were compared using ANOVA with post hoc Bonferonni procedure; a significant level of P<0.05. Cell viability of the treatments at the concentrations of 10, 20, 40 and 80 ug/ml did not significantly differ to that of non-treated cells in all the cases the test was performed (P>0.05). Cell viability was dramatically decreased at 48 and 72 h with 160 8g/ml (P<0.01), and further cell viability was also decreased at both time points by 360 8g/ml (P<0.001). The % SEM of each experimental group is indicated on each bar graph on the graph. We employed the analysis of variance (ANOVA) with Bonferonni post hoc test to determine which group was significantly different to the control group.

They also determined the impact of pre-incubation with Harpagophytum procumbens (10, 30 and 90 ug/mL) on the generation of pro-inflammatory mediators in PBMCs 2 hours prior to the challenge with LPS (1.0 ug/mL). Following 24 hrs of cell culture, supernatants were assessed using ELISA to establish the concentration of TNF-alpha, IL-6 and IL-1beta. Results of the effects of Harpagophytum procumbens (0, 10, 60, 150, LPS+1.0, LPS+10, LPS+60, LPS+150 µg/mL) on the production of pro-inflammatory mediators (TNF-alpha, Interleukin-1 beta and Interleukin-6 Pg/mL respectively) were recorded (3.96, 8.27, 4.91, 3.52, 3 And Interleukin-1β had been recorded (5.50, 10.49, 3.67, 1.50, 35.70, 16.88, 10.00 and 6.49) Pg/mL respectively. During the same time the Interleukin-6 levels were (35.00, 60.71, 53.98, 18.93, 180.01, 176.61, 144.00 and 133.58) Pg/mL respectively. The statistical analysis revealed that there was no difference between the control and 10, 30, and 90 ug/mL of Harpagophytum procumbens-treated groups as far as the concentration of TNF- 6, IL-1 in peripheral blood mononuclear cells (PBMCs) was concerned (P > 0.05). Nevertheless, PBMCs markedly up-regulated the concentration of TNF-alpha, IL-6 and IL-1 beta in medium culture when they were stimulated with LPS alone (P < 0.001). The experiments have revealed that the concentration of TNF-a, IL-6, and IL-1b were considerably enhanced in the Supernatant of LPS-stimulated PBMCs. The levels of TNF-alpha, IL-1 beta and TLR-4 were highly affected by the quantities selected among Harpagophytum procumbens (P < 0.001, P < 0.01 and P < 0.001, respectively). Anyway, there are no changes in IL-6, COX-2 [24, 27] and PGE2 in comparison with their initial levels when Harpagophytum procumbens is used. Harpagophytum procumbens [28-31] however reduced the pro-inflammatory chemical content in the cell culture media before the PBMCs activation by LPS.

Conclusions

Lastly, we evidently observed that our drug was suppressing the release and manifestation of pro-inflammatory mediators. The effects of Harpagophytum procumbens have been studied in various researches but this is the first research to show that it lowers the concentration of inflammatory mediators in the human cell lines when subjected to LPS. The key results obtained encourage the researchers to carry out more investigations on the compounds so that they can be used as anti-inflammatories. Just to be on the safer side it is always a good idea to do some extra research just to confirm that we are on the right path. It also has more research which needs to be conducted on the mechanism of action of the active ingredients in the medicine. To that we should include the study of what we find in animals with inflammation and autoimmune diseases and human beings with similar problems.

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