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Original Article

Antioxidant and Inflammatory Cytokines Regulatory Actions of Fresh Snail and Seawater Gastropods Extracts

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Abstract

Context:

Mollusca also termed mollusks. These are unsegmented, bilateral animals. Freshwater and seawater gastropods are considered rich source of bioactive molecules that possesses various therapeutic potential.

Aim:

The present work aimed to investigate the antioxidant, wound healing, and anti-inflammatory effects along with the regulation of inflammatory protein at the cellular level by apple snail (freshwater gastropod) and tibia shell (seawater gastropod) extract.

Materials and Methods:

The mass of freshwater and seawater gastropods was macerated in water.

Results:

The aqueous extract of apple snail exhibited the highest 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS) activity at 85.62% and 73.35% at 10 mg/ml, whereas the aqueous extract of seawater gastropod exhibited the highest DPPH radical scavenging activity and ABTS activity 83.53% and 76.14% at 1 mg/ml. Marine water snail aqueous extract and freshwater snail aqueous extract, each of 250 mg/kg showed 83% and 67.14% inhibition of paw edema, respectively. The cell viability study was performed by MTT assay and both the extracts showed cell viability above 97%. Both samples (freshwater snail extract and seawater gastropod extract) have good anti-inflammatory effects as they lower the concentration of tumor necrosis factor-alpha (pro-inflammatory cytokines) and increase interleukin-10 (anti-inflammatory cytokines).

Conclusion:

The present work concluded that the extracts of seawater gastropods were found to be more effective as an antioxidant and anti-inflammatory agent.

INTRODUCTION

M*ollusca* is unsegmented, soft-bodied, bilaterally animals also known as mollusks. The body of the *Mollusca* is organized into a muscular foot, a head visceral mass containing various organ systems, and a calcareous shell. The *Mollusca* has the largest species of about 100,000, and nearly, 100,000 species have not been described yet.[1] The gastropod is a large taxonomic class of invertebrates within the phylum *Mollusca* commonly known as snails and slugs.[2] Gastropods can be found abundantly in seawater ecosystems, freshwater, or estuary areas.[34567]

Freshwater and seawater organisms are considered a vast untapped resource of bioactive molecules

with enormous therapeutic potential. This has led to a growing interest in the investigation of natural products for the discovery of different activities including antioxidant, anti-inflammatory, and antimicrobial compounds.[8] The rich diversity of organisms assumes a great opportunity for the discovery of new bioactive compounds.[9] In recent times, various studies have been reported on the investigation of bioactive compounds with their pharmacological activities from mollusks.[10,11,12]

The literature revealed that a huge number of works have been carried out in other groups of organisms but only a few studies were made on mollusks.[13,14,15,16,17] The availability of *Mollusca* is too high and their utilization is extremely low compared to other marine organisms. Hence, the present investigation was carried out to explore the antioxidant and anti-inflammatory effects along with the regulation of inflammatory protein at the cellular level by apple snail (freshwater gastropod) and tibia shell (seawater gastropod) extract. The extracted solvent was concentrated under a vacuum using a rota-evaporator.

MATERIALS AND METHODS

Materials

Chemicals

1,1-diphenyl-2-picrylhydrazyl (DPPH), gallic acid, 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulphonic acid (ABTS), ascorbic acid, and potassium persulfate were purchased from S.D. Fine Chemicals, Mumbai, India.

The reagents and the sample solutions used for evaluation are methanolic solution of DPPH (1 mM), drug stock solution (0.25–10 mg/ml), ABTS solution (2 mM), and potassium persulfate solution (17 mM).

Cell line and reagents and kits

RAW 264.7 cell line was obtained by the National Center for Cell Science (NCCS) in Pune, India. Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), and phosphate buffer saline were purchased from Invitrogen (Carlsbad, USA). Lipopolysaccharide (LPS) purified from *Escherichia* (serotype 026:B6) was purchased from Sigma-Aldrich Co.(India) Enzyme-linked immunosorbent assay (ELISA) kits were purchased from RayBiotech Inc., USA.

All other chemicals and reagents used in this study were of analytical grade.

Instruments

The instruments used for the study were a UV spectrophotometer (JASCO, V-630), laboratory centrifuge (Remi motors, R4C), and digital pH meter.

Methods

Procedure for extraction of gastropod

The gastropods also termed snails and slugs are found in freshwater as well as in seawater.

The present work involved two species: *Pila globosa* commonly known as pond snails obtained from freshwater and *Tibia curta* commonly termed as beak shells collected from marine water. The freshwater snails were collected in the month of June. The cleaned snail (2 kg) mass was macerated in water (1000 ml × 3) for 7 days each cycle below 20°C. After each cycle, the extracted solvent was replaced by fresh volume. The extracted solvent was concentrated under a vacuum using a rota-evaporator.

The sea gastropods were collected in the month of January. After collection, the gastropods were rinsed with sterile seawater to remove associated debris. The mass (500 gm) was separated from shells and was macerated in water (250 ml × 3) for 7 days each cycle below 20°C. After each cycle, the extracted solvent was replaced by fresh volume. The extracted solvent was concentrated under a vacuum using a rota-evaporator.

In vitro antioxidant studies

1,1-Diphenyl-2-picrylhydrazyl radical scavenging assay

The 1 ml solution of 0.1 mM DPPH in methanol solution was added to 3 ml of different test compounds at different concentrations ranging from 0.1 to 10 mg/ml for freshwater snails and 0.01–1000 mg/ml for marine snails. Thirty min later, the absorbance was measured at 517 nm. The % inhibition and IC50 were calculated from the following equation:

2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid assay

The ABTS solution of 7 mM was prepared by dissolving ABTS in water. ABTS radical cation (ABTS+.) was produced by reacting ABTS stock solution with 2.45-mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h. The ABTS+. solution was diluted with a phosphate buffer (2 mM, pH 7.4) to achieve an absorbance of 0.8 ± 0.014 at 734 nm. The sample solutions were mixed with ABTS+. solution, and the absorbance was measured using UV–Vis spectrophotometer at 734 nm after 1 min. Phosphate buffer solution was used as a blank. The % radical-scavenging activity of the samples was determined using the formula

Anti-inflammatory activity

Anti-inflammatory activity was measured using a carrageenan-induced rat paw edema assay. Rats were allocated into seven groups (six animals each) and fasted overnight the day before the day of the experiment. Inflammation was induced by intraplantar administration of 0.1 mL of carrageenan solution (3% w/v in saline) in the right hind paw of each rat of all the groups except group A. Animals of group B/C, D/E, and F/G were treated with the single dose of control receiving only carrageenan, standard drug receiving group: diclofenac (10 mg/kg), freshwater snail aqueous extract (50 mg/kg), freshwater snail aqueous extract (250 mg/kg), marine snail aqueous extract (50 mg/kg), and marine water snail aqueous extract (250 mg/kg), respectively, 30 min before carrageenan injection. The anti-inflammatory activity was indicated in terms of paw edema volume (in ml) which was measured using a plethysmometer for 5 h.

Evaluation of the effect of extracts on anti-inflammatory cytokines

Cell culture and culture conditions

RAW 264.7, a mouse macrophage cell line was obtained from National Centre for Cell Science (NCCS, Pune, India). RAW 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 μg/ml streptomycin, and 50 units/ml penicillin. The cells were incubated at 37°C in the presence of 5% CO_2 and were subcultured every 2 days.

Cell viability assay

The viability of the cells was evaluated by (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) colorimetric assay.[18] The RAW 264.7 cells were seeded into a 96-well culture plate at a density of 5×10^4 cells/well and incubated overnight at 37°C and 5% CO_2 for attachment. The cells were then treated with a concentration range from 50 to 1000 μg/ml of both samples with (1 μg/ml) or without LPS and incubated for 24 h. After incubation, the culture medium was removed, and 100 μl of fresh DMEM and 20 μl of MTT (5 mg/ml in PBS) solution were added to each well. Following 4-h incubation in dark, the media was discarded again and 100 μl of DMSO was added to each well for the solubilization of formazan deposits. The optical density of the cells at 570 nm was measured using an ELISA plate reader (Bio-Rad Laboratories, CA, USA), and the experiment was carried out in triplicate.

Measurement of pro-inflammatory and anti-inflammatory cytokines (Tumor Necrosis Factor-alpha and interleukin-10) production

The RAW 264.7 cells were seeded at a density of 2 × 104 cells/well in 24-well culture plates and incubated for 24 h at 37°C and 5% CO_2 for adherence. The adhered cells were incubated for 24 h, with the indicated concentrations of test samples (500 μg/ml) in the absence or presence of LPS (1 μg/ml). The cell culture supernatant was harvested after 24 h of incubation of cells with LPS and samples. These supernatants were tested for quantitation of pro-and anti-inflammatory cytokines (tumor necrosis factor α [TNF-alpha] and interleukin-10 [IL-10]) using a mouse-specific enzyme immune assay kit (RayBiotech Inc., USA) according to manufacturer's instructions.

Briefly, the ELISA plates (96 well) were coated with specific mouse TNF-α, IL-10 antibodies (100 μl/well), and incubated at 4°C for overnight. The assay diluents (200 μl/well) were used to block the nonspecific protein-binding sites present in the plate. Immediately, 100 μl of culture supernatant or respective standard was added into the suitably coated wells and incubated at room temperature for 2 h. After incubation, the plates were washed five times thoroughly with wash buffer (phosphate-buffered saline [PBS] containing 0.05% Tween-20). About 100 μl of detecting solution (detection antibody and streptavidin-horseradish peroxidase) was added to each well.

The plates were properly covered with a plate sealer and incubated for 1 h at room temperature and again washed five times thoroughly using wash buffer. 100 μl of the substrate solution, tetramethyl benzidine (TMB) was added to each well and the plate was further incubated (without plate sealer) for 30 min in the dark at room temperature. Finally, 50 μl of stop solution (2N H_2SO_4) was added to each well. ELISA results were recorded at 450 and 570 nm with an ELISA reader (Bio-Rad Laboratories, CA, USA). The concentrations were determined for three wells for each cytokine, and values were derived from the standard curve and expressed as pg/ml.

RESULTS

Yield of extracts

The dried extract of freshwater snail yielded 13.1%, 262.0 g out of 2000 g, and seawater snail was extracted to yield 35 g (7%) extract out of 500 g of mass. Both extracts were stored in a refrigerator

for further analysis.

In vitro antioxidant studies

The DPPH scavenging activity and ABTS of aqueous extract of apple snail are shown in Figures 1 and 2 and of seawater gastropod in Figures 3 and 4.

F1-8
Figure 1:
DPPH radical scavenging assay of apple snail. DPPH: 1,1-Diphenyl-2-picrylhydrazyl

F2-8
Figure 2:
ABTS assay of apple snail. ABTS: 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid

F3-8
Figure 3:
DPPH radical scavenging assay of AO tibia gastropod. DPPH: 1,1-Diphenyl-2-picrylhydrazyl

F4-8
Figure 4:
ABTS assay of AO tibia gastropod. ABTS: 2,2'-azinobis-(3-ethylbenzothiazoline)-6-sulfonic acid

The aqueous extract of apple snail exhibited the highest DPPH radical scavenging activity and ABTS activity at 85.62% and 73.35% at 10 mg/ml, whereas the aqueous extract of seawater gastropod exhibited the highest DPPH radical scavenging activity and ABTS activity at 83.53 and 76.14% at 1 mg/ml. The IC50 concentration of DPPH radical scavenging assay and ABTS assay of methanol extract of AO apple snail were 4.03 and 6.27 mg/mL, respectively. The IC50 concentration of DPPH radical scavenging assay and ABTS assay of methanol extract of AO apple snail were 489.07 and 552.32 µg/ml, respectively. The highest percentage of inhibition of both extracts is similar, but the IC50 values are very much different. Freshwater snail extract exhibited IC50 at approximately 4.02 mg/ml and 6.27 mg/ml for DPPH and ABTS activity, respectively. However, seawater snail extract showed IC50 values for DPPH and ABTS activity at 489.07 µg/ml and 552.32 µg/ml, respectively. Thus, it was clear that seawater snail was comparatively more potent than freshwater snail extract.

There was dose-dependent significant reduction in carrageenan-induced rat paw edema over a period of 4 h of each test sample. Significant anti-inflammatory activity was noted with 250 mg/kg of both extracts at different time intervals (i.e. 1 h, 2 h, 3 h, 4 h, and 5 h) and a significant percent reduction in edema volumes was noted [Figure 5]. Marine water snail aqueous extract (250 mg/kg) showed 83%, whereas freshwater snail aqueous extract (250 mg/kg) exhibited approximately 67.14%. The marine snail was more effective in lowering inflammation than freshwater snail extract.

F5-8
Figure 5:
Percentage decrease in paw volume at time intervals

Effect of gastropod extracts on the levels of pro and anti-inflammatory cytokine

Chronic inflammation may develop following acute inflammation and may last for weeks or

months, and in some instances, for years. The macrophages contribute to the inflammatory process by chronically elaborating low levels of IL-1 and TNF which are responsible for some of the resulting clinical symptoms such as anorexia, cachexia, fever, sleepiness, and leukocytosis.

TNF-alpha shares an important inflammatory property with IL-6 and IL-11, i.e., the induction of acute-phase reactant protein production by the liver. TNF-alpha and IL-1 further exert secondary inflammatory effects by stimulating IL-6 synthesis in several cell types. IL-6 then mediates its own effects and those of TNF-alpha and IL-1 in inducing fever and the acute-phase response, thereby perpetuating the inflammatory response through a cascade of cytokines with overlapping properties.

IL-10 is also referred to as B-cell-derived T-cell growth factor and cytokine synthesis inhibitory factor because it inhibits interferon-gamma (IFN-γ) production by activated T-cells. IL-10 is produced by a variety of cell types, including CD4 + T-cells, activated CD8 + T-cells, and activated B cells.

Since IL-10 can be produced by TH2 cells and inhibits TH1 function by preventing TH1 cytokine production (such as IFN-γ), IL-10 is considered a T-cell cross-regulatory factor and has thus been referred to as an “anti-cytokine.”

The present study determines the potency of gastropod extracts as anti-inflammatory agents. This study could further clarify whether the intended action of each extract is through the mechanism of lowering systemic inflammation. Thus, we examined the anti-inflammatory effect of both gastropod extracts by ELISA study in RAW 264.7 cell lines.

Macrophages play major roles in innate immunity and they recognize pathogen-associated molecules such as LPS (a bacterial endotoxin) and trigger an innate immune response through toll-like receptor (TLR) signaling. Due to the binding of LPS to the TLR4 receptor, an intracellular tyrosine kinase system is upregulated, which in turn leads to stimulation of the transcriptional factor NF various inflammatory mediators such as TNF-α, IL-1α, and IL-6. The inhibition of adverse macrophage activation or selectively neutralizing the overproduction of macrophage products was recommended as a promising therapeutic route against diverse inflammatory conditions.

Evaluation of the effect of both extracts on cell viability of RAW 246.7 by MTT assay

The cell viability study was performed by MTT assay and it was clearly understood that both the extracts in the concentration range of 50–1000 μg/ml showed above 97% cell viability [Figure 6]. This depicts that the extracts can be effective as anti-inflammatory agents. Thus, for the ELISA study, 500 μg/ml concentration (median concentration) was selected.

F6-8
Figure 6:
Effect of extracts at different concentration on cell viability by MTT assay

Effects of freshwater gastropod extract on pro-inflammatory and anti-inflammatory cytokines production

The maximum suppressive effect of freshwater gastropod extract on pro-inflammatory cytokines, TNF-α evaluated was 927.22 pg/ml (20.64%). Moreover, the anti-inflammatory cytokine IL-10 level was elevated by extract up to 179.44 pg/ml which was at par with the normal cells. The test samples and LPS results were compared, and the percentage was calculated.

Effects of seawater gastropod extract on pro-inflammatory and anti-inflammatory cytokines production

The current study showed that seawater gastropod extract lowered the pro-inflammatory cytokines up to 520 pg/ml (50.07%) of TNF-α as compared to cells treated with LPS and increases the anti-inflammatory cytokine (IL-10) by 150 pg/ml (55.10%) shown in Figures 7 and 8. The test samples and LPS results were compared, and the percentage was calculated.

F7-8
Figure 7:
Effect of samples on TNF-α cytokine production. TNF-α: Tumor necrosis factor α

F8-8
Figure 8:
Effect of samples on IL-10 cytokine production. IL-10: Interleukin-10

In both the test samples, it was very well observed that both samples have good anti-inflammatory effects as they lower the concentration of TNF-α (pro-inflammatory cytokines) and increase IL-10 (anti-inflammatory cytokines). In comparison to both samples, seawater gastropod extract was effectively more active as an anti-inflammatory agent than freshwater snail extract.

DISCUSSION

The yield of freshwater snail extract was found to be more when compared with marine water extract.

The methanolic and aqueous extracts of freshwater snails and seawater gastropods were studied for antioxidant activity (DPPH and ABTS). The highest percentage of inhibition of both extracts is similar, but the IC_{50} values are very much different. Thus, it was clear that seawater snail was comparatively more potent than freshwater snail extract. Marine water snail aqueous extract and freshwater snail aqueous extract, each of 250 mg/kg showed 83% and 67.14% inhibition of paw edema, respectively. The results concluded that the marine snail was found to be more effective in lowering inflammation than freshwater snail extract.

The cell viability study was performed by MTT assay and both the extracts showed cell viability above 97%. Both samples (freshwater snail extract and seawater gastropod extract) have good anti-inflammatory effects as they lower the concentration of TNF-α (pro-inflammatory cytokines) and increase IL-10 (anti-inflammatory cytokines). The extract of seawater gastropod was found to be a more effective anti-inflammatory agent than freshwater snail extract.

CONCLUSION

At the site of the inflammation, inflammatory cells release a variety of reactive substances, intensifying oxidative stress.[19] Reactive oxygen and nitrogen species, on the other hand, can start an intracellular signaling cascade that increases the production of pro-inflammatory genes.[2021] Thus, oxidative stress and inflammation are closely related pathophysiological processes that are intertwined. In reality, experimental studies demonstrate the co-existence of oxidative stress with low-grade chronic inflammation in a number of chronic diseases, including diabetes complications, cardiovascular and neurodegenerative disorders, alcoholic liver disease, and chronic kidney disease.[22232425]

Thus, with our study, we can conclude that the extracts of seawater gastropods can be a good choice for integrated extract as an antioxidant and anti-inflammatory agent for various human health benefits.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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