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Intracellular reactive oxygen species scavenging potential of *Benincasa hispida* Cogn. confection

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Unchecked levels of Reactive Oxygen Species (ROS) are known contributors in numerous health issues like metabolic disorders, neurological disorders and cancers. A traditional herbal preparation, *Benincasa hispida* Confection (BHC) is hypothesized to balance the levels ROS because of the presence of inherent antioxidative phytocomponents. However, the specific mechanisms underlying BHC purported ROS scavenging effects at cellular level have remained unexplored. We prepared BHC, and profiled its antioxidative molecules through HPLC analysis. Preparation method included *B. hispida* pulp base mixed with sugar candy and various medicinal herbs as key ingredients to enhance its taste and palatability. The ability to scavenge intracellular ROS was investigated using 2',7'-dichlorofluorescin-diacetate (DCF-DA) assay after ensuring the cell viability upon treatment with the extract of BHC. BHC was rich in previously known antioxidant molecules and was able to quench intracellular ROS. *B. hispida* also contributed to its ROS quenching abilities along with other ingredients. This study provides valuable insights into the therapeutic utility of BHC and advocates that consumption of BHC might protect the individuals from the ROS mediated oxidative stress and associated diseases.

Keywords: *Benincasa hispida*, Cell viability, Metabolic diseases, Oxidative stress, Reactive oxygen species

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When there is an excess in the generation and accumulation of reactive oxygen species (ROS) beyond the innate antioxidant defense mechanisms of the body, oxidative stress ensues. This condition is associated with the impairment of the normal functioning of biomolecules, including lipids, proteins, and DNA, thereby contributing to the onset of diverse health issues such as disorders related to metabolism, neoplasms, and neurodegenerative diseases[1-3]. While ROS acts as a trigger to initiate health disorders, an indiuction of ROS through a specialized approach is emerging as a new technique to target cancers[4,5].

The overproduction of ROS not only results in the irreversible oxidation and peroxidation of proteins, lipids, and glycans, but in instances where protective antioxidant systems are unable to regulate them effectively, it can lead to the manifestation of various diseases or cell death. Conversely, localized low levels ROS play a crucial role in modulating key transcription factors such as NF-B/I-B, Nrf2/KEAP1, AP-1, p53, HIF-1, and PTK/PTP. Moreover, these ROS levels are essential for maintaining cellular homeostasis (PI3K-AKT-mTOR, PTK/PTP, and MAPK/ERK), impacting various cellular functions, including apoptosis, migration, differentiation, and proliferation[6].

Confection or Avaleha, a multi-ingredient, semi-solid dosage ayurvedic formulation is well known for its acceptability and palatability. The main constitute of *Benincasa hispida* Confection (BHC) is *Benincasa hispida*[7], also called as Shweta petha, ash gourd, white pumpkin, winter melon. *B. hispida* also known as Kushmand is one of the main ingredients of BHC and possesses many therapeutic properties owing to which it has been used in many therapeutic

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Abbreviations
Herbal plants *Benincasa hispida*, BHC: *Benincasa hispida* Confection, ROS: Reactive Oxygen Species, DCFDA: 2'-7'-Dichlorodihydrofluorescein diacetate, ICP-AES: Inductively Coupled Plasma Atomic Emission Spectroscopy.

preparations. An initial reference to BHC is found in 'Ashtanga Sangraha' (Ayurvedic Indian literature), which described its beneficial role in coughs, hiccups, fever, dyspnea, bleeding disorders, wound, emaciation, thorax injuries, promoting brain and memory and improves physical strength. *B. hispida* fruits are traditionally used as a diuretic, laxative, cardiotonic and find their usefulness in management of varios respiratory, urinary, gastrointestinal conditions[8,9]. BHC is included in the 'Essential Drug List' recommended by the Ministry of Ayush, Government of India[10].

B. hispida is recognized as functional food due to the presence of various health-promoting phytonutrients[11]. Numerous phytochemicals, including triterpenoids, flavonoids, glycosides, saccharides, carotenes, vitamins, β-sitosterin, and uronic acid, are abundant in *B. hispida*. This plant's fruit is beneficial for treating a variety of illnesses, such as cancer, diabetes, heart disease, and inflammation. *B. hispida* fruit extract in a water-in-oil cream has shown antioxidant activity, suggesting that it may be able to postpone the signs of ageing[12–14].

Few studies have provided a detailed description of the *B. hispida* activities. It was recently reported to exhibit free radical scavenging capability on skin[15].

Intracellular ROS scavenging activity of BHC has not been documented to date. In the first of this kind study, we prepared BHC, established its biochemical profile and checked its role in the intracellular ROS scavenging in cell lines.

Materials and Methods

Raw materials and chemicals

Raw drug materials were procured from a market in Varanasi, Uttar Pradesh, India, and were certified for authenticity by the Botany department at Banaras Hindu University, Varanasi. MTT reagent and DCFDA (2'-7'-Dichlorodihydrofluorescein diacetate) reagents were obtained from Sigma. Cell culture media and other products were purchased from HiMedia, unless specified otherwise.

Preparation of BHC

BHC (Kushmand Avaleha) was prepared in accordance with the guidelines specified in 'Sharangdhar Samhita,' Madhyam Khand, 8/1-3, within the pharmaceutical facilities of the Department of 'Rasa Shastra and Bhaishjya Kalpana,' Faculty of Ayurveda, Institute of Medical Sciences, Banaras Hindu University. The formulation utilized *B. hispida*, procured from the local market. *B. hispida* was finely chopped into small pieces following the removal of the outer skin, and was boiled with four volumes of water. The boiled pulp was strained through a cleansed, dried cloth, separating the liquid component (decoction). The resultant paste was then fried in clarified butter until it achieved a brown hue.

Subsequently, the fried paste of *B. hispida* was introduced to a mixture of sugar candy (mishri) along with the separated decoction. The mixture was gently heated until the desired appearance of three threads when a drop of the mixture was placed between the thumb and index finger (Signs of perfectly prepared confection). Finally, additives (Prakshepa dravyas mentioned in Table 1) were added into the preparation at the lukewarm stage. Upon reaching the room temperature, honey was added into the preparation and thoroughly blended. The methodology has been detailed in the Figure 1. All the ingredients have been enlisted in the Table 1.

Characterization of the raw drugs

Heavy metal content analysis

The heavy metal analysis was carried in Indian institute of Technology, Bombay, Powai, Mumbai using Inductively Coupled Plasma Atomic Emission

Table 1 — Ingredients and composition by parts of the *Benincasa hispida* Confection

Ingredients	Botanical Name	Family	Part used	Quantity
Kushmand	*Benincasa hispida*	Cucurbitaceae	Fruit	200 parts
Pippali	*Piper longum*	Piperaceae	Fruit	8 parts
Shunthi	*Zingiber officinale*	Zingiberaceae	Rhizome	8 parts
Jiraka	*Cuminum cyminum*	Umbelliferae	Fruit	8 parts
Aila	*Eletteria cadamomum*	Zingiberaceae	Seed	1 part
Maricha	*Piper nigrum*	Piperaceae	Fruit	1 part
Tvaka	*Cinnamonum zeylanicum*	Lauraceae	Stem bark	1 part
Dhanyaka	*Coriandrum sativum*	Umbelliferae	Fruit	1 part
Tejpatra	*Cinnamomum tamala*	Lauraceae	Leaf	1 part
Honey				32 part
Clarified Cow Butter				16 part

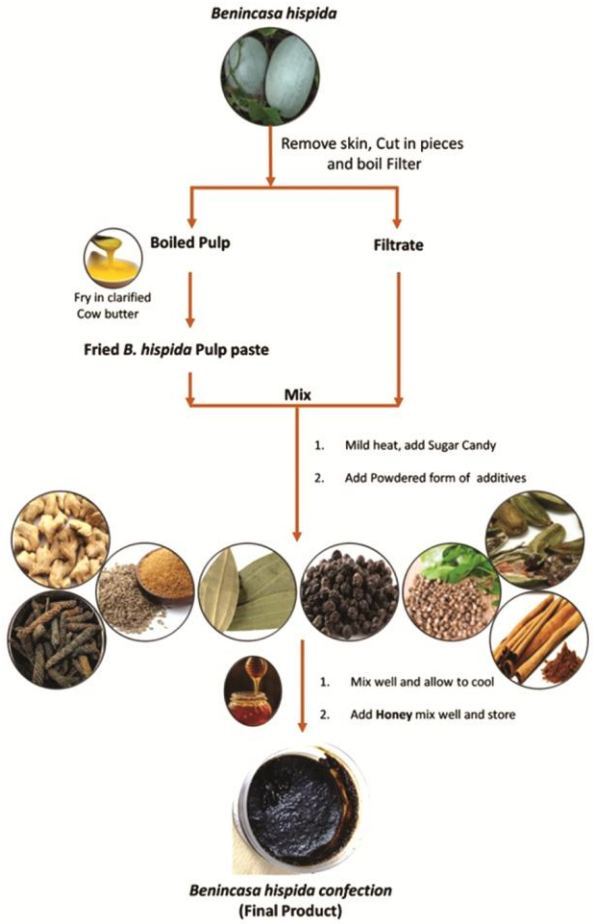

Fig. 1 — Methodology for the preparation of BHC

Spectroscopy (ICP-AES). Each sample was tested thrice. The limits of quantification were 3 ppm for mercury, arsenic, cadmium and 10 ppm for lead. Sample was prepared by using diluted aqua regia and concentration of sample was kept at 1 ng/mL.

Determination of pH of BHC

BHC was resuspended in milliQ water and was shaken vigorously for 5 min. The aqueous extract was filtered using and the pH of the filtered extract was pH was recorded using a digital pH meter (Labmann). The sample was measured in triplicates and mean value was determined.

Characterization of the BHC phytoconstituents

For this investigation, BHC extract or extract of the individual components was prepared using the Soxhlet extraction method.

High performance liquid chromatography (HPLC) analysis

The HPLC analysis of the samples (BHC and individual components) was done as previously described[13]. Briefly, methanolic extract of BHC was prepared and collected in a fresh Eppendorf tube, followed by centrifugation at 10,000 rpm for 15 min. The supernatant was fractionated using ethyl acetate (1:1). The organic fraction was then collected and subjected to three successive re-fractionations with ethyl acetate to ensure collection of the organic components present in BHC. The final pellet obtained was solubilized in HPLC grade methanol (50%) and analyzed using HPLC for qualitative and quantitative metabolite analysis.

The HPLC system from Shimadzu LC-10A, Japan, equipped with a dual pump LC-10A binary system, UV detector SPD-10A, and a Phenomenex (Torrance, USA) C18 column (RP-Hydro, 4 µm, 250 mm × 4.6 mm) was employed for sample analysis. Data integration was performed using Shimadzu Class LC Solutions software. Compound separation was achieved using a linear gradient program of acetonitrile/water (1:1 v/v) with 1% acetic acid, initiating with 18% acetonitrile and switching to 32% in 15 min, and finally to 50% in 40 min[16]. The solvent flow rate was adjusted to 1 mL/min. The experiment was concluded after 30 min, and the optical density of the eluents was measured at 254 nm, with subsequent comparison of sample peaks to their corresponding standard peaks.

Preparation of extract for cell-based assays

500 mg of the BHC was dissolved in 10 mL of 80% ethanol (50 mg/mL stock). BHC was mixed well by pipetting several times. The extract was kept at 37°C for 1 h followed by mixing. The extract was spun at 4000 rpm for 10 min and the supernatant was used for subsequent experiments. The extract was passed through a 0.45 µM filter before storing at 4°C for subsequent experiments. The protocol follows a similar study for extract preparation[17]. *B. hispida* extract was obtained by boiling *B. hispida* in water, which was used in the preparation of the confection from the same batch. The extract was considered to be of 100% potency.

Cell culture

HEK cell line was procured from the National cell Line repository, National Center for Cell Science (NCCS) Pune, Maharashtra India. HEK Cells were cultured in high glucose-Dulbecco's Modified Eagle's Medium (DMEM), with addition of 10% fetal bovine serum, 2.0 mM glutamine, 1% antibiotic concentration in a CO_2 incubator (5% CO_2) under

humid environment at 37°C. HEK cells were subcultured at reaching 80% confluence.

Cell viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to check the cell viability[18]. HEK cells were plated at a density of 5000 cells per well in 100 µL of DMEM. Cells were allowed to grow overnight in the CO_2 incubator. After 12 h, cells were incubated with various concentrations of the BHC (1000, 333, 111, 37, 12.3, and 0 µg/mL) and the *B. hispida* extract (10%, 5%, 2.5%, 1.25%, 0.625% and 0%) for 24 h. Briefly, cells were mixed with 10 µL of the MTT (5 mg/mL stock) for 2 h in a CO_2 incubator at 37°C. Supernatant culture media was decanted and 100 µL of DMSO was added to each well to dissolve intracellular formazan crystals. The absorbance was recorded at 570 nm on a microplate reader (Biotek Synergy HT).

Intracellular ROS determination using 2',7'-Dichlorofluorescein diacetate (DCFDA)

Intracellular ROS was determined using 2',7'-Dichlorofluorescein diacetate as described in a previous protocol[19]. Human Embryonic Kidney (HEK) cells were seeded in a 96-well plate at a density of 10,000 cells/well in 100 µL volume. After overnight growth, cells were incubated with 100 µg/mL of BHC and 1% of the *B. hispida* extract in triplicates for 24 h. H_2O_2 and cells alone served as respective controls for the experiment, treatment with H_2O_2 of the respective groups was done for 3 h prior to the addition of DCFDA. Cells were washed with prewarmed DMEM once and 10 µM DCFDA (Sigma Aldrich) was added to each well for 30 min in a humidified CO_2 incubator at 37°C in dark. After the staining, the cells were washed with DMEM once and twice with prewarmed 1X PBS. The plate was read using Microplate Reader (Biotek Synergy HT) in the green channel (excitation 485 nm and emission 530 nm).

Data analysis

The data were analyzed using excel and the GraphPad software was used to calculate IC-50.

Results

Organoleptic drug characteristics

The final product was prepared using the described methodology (Fig. 1). The final product had consistency of a typical ayurvedic confection preparation. It had a thick semisolid consistency with blackish brown appearance and sweet-astringent taste. The odour had a hint of ingredient spices and it was soft sticky to touch.

Heavy metal study

ICP-AES analysis was performed on the BHC dissolved in diluted aqua regia to determine the limits of heavy metals (Hg, Pb, As, Cd) in the sample. The results of triplicate demonstrated that the tested heavy metals were below the detection limits of the equipment and were insignificant.

Determination of pH of BHC

The pH value aqueous extract was determined using a digital pH meter in triplicates. The mean value of the pH of the aqueous extract was found to be 6.18, which is close to the neutral pH and is acceptable as edible.

BHC is a typical confection rich in phytoconstituents with antioxidative activities

The phytoconstituents analysis of the methanolic extract of the BHC and its individual components (Table 1) was done using HPLC method to look for the presence of anti-oxidative bioactive phytochemicals. As expected, the HPLC profile showed that BHC exhibited presence of several key phyto-components (Supplementary Table S1-8) implemented in ROS scavenging such as Trans Cholorogenic acid, Tannic acid, Synafic acid, Salicylic acid, Rutin, Quercetin, p-Coumaric acid, Gallic acid, and Feuralic acid. Majority of these were also detected in the individual components (Fig. 2).

BHC and *B. hispida* extract are non-toxic to human cells

In order to establish the toxicity and safety values for cell-based studies, well acknowledged MTT based cell viability assay was employed. HEK cells treated with BHC (Fig. 3a) and extract (Fig. 3b) did not show any toxicity at various concentration treated. This shows that these preparations are safe to consume.

BHC ameliorates intracellular ROS burden

Finally, in order to score the effects of BHC on intracellular ROS scavenging, HEK cells were treated with the BHC and extract. As expected, HEK cells treated with 100 µg/mL BHC showed reduction in ROS induced by H_2O_2. Extract also showed a reduction, which however was inferior to the BHC.

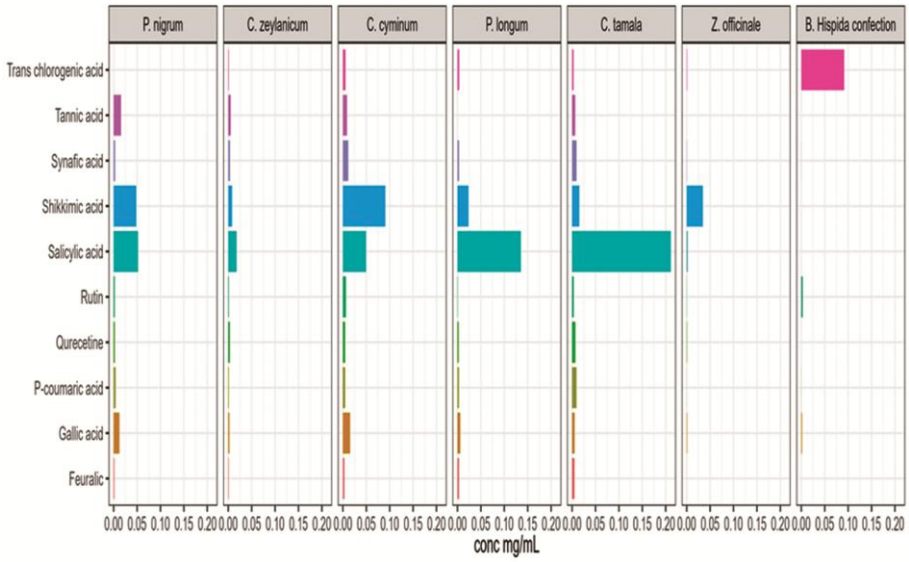

Fig. 2 — Phyto-constituent analysis of the BHC and its raw materials. HPLC analysis of the materials along with standards of the signature antioxidant compounds showed the presence of Trans Cholorogenic acid, Tannic acid, Synafic acid, Salicylic acid, Rutin, Quercetin p-Coumaric acid, Gallic acid, Feuralic acid

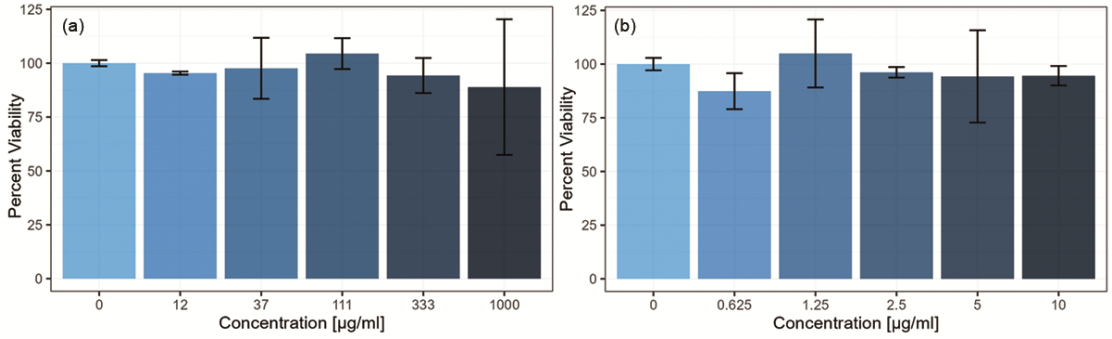

Fig. 3 — BHC and *B. hispida* extract are non-toxic for cells. HEK cells were treated with different concentration of either BHC or *B. hispida* extract. There was no detection of a significant growth inhibition in all the concentrations tested. Means of triplicate values are plotted with standard deviation as error bars

Discussion

Chronic and acute oxidative stress lies at the foundation of a number of ailments such as cancers, aging, and neurodegenerative diseases[1–3,6,20]. While the cause of the generation of ROS are numerous[1] , they have huge potency to cause damage to cellular macromolecules such as nucleic acids, proteins and lipids which are deteriorating for cellular functioning and therefore culminating into the illness[6]. BHC, characterized by its sweet and astringent properties, presents an appealing option for consumption as a health nutrition for rejuvenation. Given the inherent antioxidative properties of its constituents, particularly those of *B. hispida*, a comprehensive examination of ROS modulation profiles becomes imperative.

Initial investigation undertaken for the presence of signature anti oxidative molecules and verified their appearance in individual drug components as well as the finished product BHC. The analysis revealed no detectable limits of the recommended heavy metal thresholds for cadmium (Cd), lead (Pb), arsenic (As), and mercury (Hg). Notably, BHC and the extract of *B. hispida* exhibited non-toxicity to in vitro cells, even at higher concentrations, affirming the safety of the drug.

Intracellular ROS can harm cells internally by causing damage to various organelles and macromolecules including DNA as suggested earlier and hence can have greater implication in disease initiation and progression. *B. hispida* pulp based nanoparticles were recently shown to exhibit potent

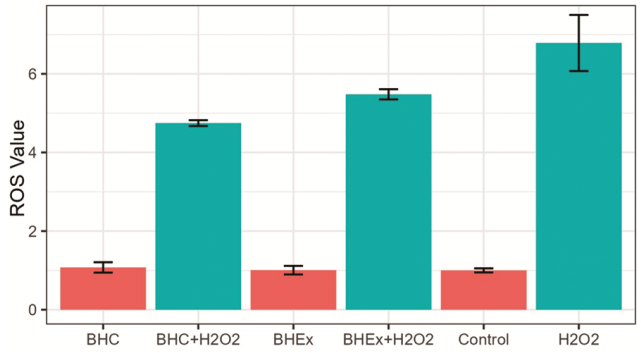

Fig. 4 — BHC as scavenger of intracellular ROS. HEK cells were treated with 100 µg/µL BHC extract and 1% *B. hispida* extract. Both BHC and *B. hispida* extracts decreased the ROS and hence display ROS protective effects. Y axis has been normalized to the control for comparison. BHC: *Benincasa hispida Confection*, BHEx: *Benincasa hispida* extract, H_2O_2: Hydrogen Peroxide

anti oxidant and anti inflammatory activities under in vitro condition[21]. As expected, BHC exhibited intracellular ROS scavenging activity (Fig. 4). Key antioxidants such as trans cholorogenic acid, tannic acid, synafic acid, salicylic acid, rutin, quercetin, p-coumaric acid, gallic acid, and feuralic acid found in the BHC are anticipated to play a major role as they are known to exhibit antioxidative capabilities. In addition to the antioxidative properties these compounds have also been documented to exhibit anti-inflammatory and immunomodulatory activities (Supplementary Table S1-8). *B. hispida* extract also exhibited a reduction in ROS levels, albeit to a lesser extent compared to BHC. This discrepancy suggests that the additional components, or "Prakshep Dravyas," in the formulation contribute to the antioxidative activity of BHC. Collectively, these findings imply that the consumption of BHC may confer greater benefits in oxidative stress-related diseases compared to *B. hispida* alone.

Conclusion

Rasayans/rejuvenators, such as BHC, are acknowledged for their propensity to progressively enhance health. Nonetheless, the precise mechanisms underlying their mode of action remain undetermined. Consequently, a comprehensive investigation is imperative to elucidate the molecular mechanisms governing their effects and to identify the principal biomolecules predominantly responsible for intracellular/extracellular reactive oxygen species (ROS) scavenging activity, either individually or in combination. Subsequent research endeavors should involve meticulous animal studies followed by rigorous clinical trials. This strategic approach of consuming plant-based nutritional supplements is advocated due to comparatively lesser side effects. While, the whole preparation contributed to the intracellular ROS quenching ability, more directed studies focusing on comparative analysis of individual components for ROS quenching should also be done.

Supplementary Data

Supplementary data associated with this article is available in the electronic form at https://nopr.niscpr.res.in/jinfo/ijtk/IJTK_24(1)(2025)16-22_SupplData.pdf

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Conflict of Interest

The authors declare no conflict of interest related to this study.

Author Contributions

Conceptualization: KP, NG; Data curation: KP, NG; Formal analysis; Funding acquisition: KP, NG; Investigation: PK, SP; Methodology: PK, SP; Project administration: KP, NG; Resources: KP, NG; Software: PK, SP; Supervision KP, NG; Validation KP, NG; Visualization: PK; Roles/Writing - original draft: PK; and Writing - review & editing: KP, NG.

Data Availability

Supporting data has been provided as a supplementary material as an additional file.

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