

COMPARATIVE ANALYSIS OF MACRONUTRIENTS IN *ASTERIAS RUBENS* EXTRACT, TINCTURE AND ULTRA HIGH DILUTIONS

Berlina Terrence Mary. D¹, Winston Vargheese V^{2*}, Krishna Kumari Amma³, Britto Wilbert Dhas. D⁴, Bencitha Horrence Mary. D⁵

¹Ph.D. Research Scholar, Department of Materia Medica, Sarada Krishna Homeopathic Medical College, (Affiliated to The Tamil Nadu Dr. M.G.R. Medical University, Chennai), Kulasekharam, Kanyakumari District, Tamilnadu, India, Email Id: berlinbright@gmail.com

²Professor, Department of Materia Medica, Sarada Krishna Homeopathic Medical College, (Affiliated to The Tamil Nadu Dr. M.G.R. Medical University, Chennai), Kulasekharam, Kanyakumari District, Tamilnadu, India, Email Id: drwinstonv@gmail.com

³Professor, Department of Materia Medica, Sarada Krishna Homeopathic Medical College, (Affiliated to The Tamil Nadu Dr. M.G.R. Medical University, Chennai), Kulasekharam, Kanyakumari District, Tamilnadu, India, Email Id: drerkrishnakumari@gmail.com

⁴Professor, Department of Materia Medica, Venkateswara Homeopathic Medical College and Hospital, (Affiliated to The Tamil Nadu Dr. M.G.R. Medical University, Chennai) Porur, Chennai, Tamilnadu, India, Email Id: drdbritto@gmail.com

⁵Associate Professor, Department of Paediatrics, Sarada Krishna Homeopathic Medical College, (Affiliated to The Tamil Nadu Dr. M.G.R. Medical University, Chennai), Kulasekharam, Kanyakumari District, Tamilnadu, India, Email Id: dr.bencitha@gmail.com

ABSTRACT

Marine organisms are recognized as valuable sources of biologically active compounds with significant therapeutic potential. While marine echinoderms are recognized as rich sources of structurally diverse, bioactive macromolecules, there remains a critical gap in scientific literature regarding the definitive biochemical composition of *Asterias rubens* across its various traditional and ultrahigh-dilution preparations. *Asterias rubens* has a long-standing history of clinical use in complementary and homeopathic medicine for treating cardiac dysfunctions, haemorrhagic tendencies, and malignant conditions. This study aimed to perform a quantitative comparative evaluation of primary macronutrients—specifically proteins, carbohydrates and lipids—in the crude ethanol extract, mother tincture(Φ), and ultrahigh dilutions 3X, 12C and 30C of *Asterias rubens*. Specimens collected from Tuticorin, Tamil Nadu, India, were authenticated, dried, and processed. While the crude ethanol extract was made through Soxhlet extraction, the mother tincture (Φ) and potencies of 3X, 12C, and 30C were made using conventional Hahnemannian techniques of repeated succussion in the liquid state. The total amount of protein, carbohydrate, and lipid contents was determined quantitatively through UV-Visible Spectrophotometric technique using Lowry, Anthrone, and Sulfo-Phospho-Vanillin techniques, respectively. It was seen that ethanol extract had maximum amounts of carbohydrate and lipid contents, validating the high lipid content of the organism. On the contrary, lower ultra-high dilutions had relatively more amounts of protein content whereas there was a progressive reduction in lipid and carbohydrate contents with increase in ultra-high dilutions. It was evident from the results that despite being at ultra-high dilution levels (30C), which exceed the classical Avogadro Limit in mathematics, *Asterias rubens* preparations had measurable amounts of primary macronutrients, thereby validating that molecular characteristic is retained during the process of potentization. These findings support modern material science hypotheses that homeopathic potentization acts through the creation of complex, stable nano colloidal systems.

KEYWORDS: *Asterias rubens*; Phytochemical analysis, Ultrahigh Dilutions; Nanoparticles; Potentization;

1. INTRODUCTION

Asterias rubens, commonly known as the common starfish or red starfish, is not used in conventional medicine or traditional food cultures in the same way as medicinal herbs. The well-documented historical and traditional use of *Asterias rubens* is in homeopathy, where it was first introduced and "proven" (tested) by Dr. Petros in 1850. In this tradition, a tincture is prepared using the whole, live red starfish.

In homeopathy, medicines derived from animal sources are considered highly potent and are traditionally used in various pathological conditions.^(1,2) *Asterias rubens*, a marine echinoderm, is a well-documented homeopathic remedy indicated in disorders related to cardiac dysfunctions, haemorrhagic tendencies, and malignant conditions.^(1,3) Despite its long-standing clinical application, there is limited scientific data supporting its biochemical composition, especially across different ultra-high dilutions.⁽³⁾

Phytochemicals refer to bioactive plant compounds that occur naturally in plants and marine organisms, playing an important role in their therapeutic value.^(4,5) They are effective in providing protection to organisms against oxidative stress, microbial infections, and damage. Quantitative phytochemical analysis is important as it provides details about the levels and occurrence of key biochemical components like proteins, carbohydrates, and lipids, which are necessary in correlating biological activity with chemical structure.⁽⁶⁾ Marine organisms have recently been found to be excellent sources of structurally diverse and biologically active compounds due to their adaptive environment.^(5,7) Echinoderms, especially starfish, contain a number of bioactive chemicals like proteins, carbohydrates, lipids, steroids, and glycosides

which display antimicrobial, antioxidative, anti-inflammatory, and anticancer activity.^(8,9) Proteins are necessary for repair of cell structures and enzymes whereas carbohydrates act as an energy source and lipids maintain cellular membrane structure.⁽¹⁰⁾ Biochemical composition of marine-derived substances is the foundation of traditional and complementary medical systems.⁽¹¹⁾

The present study focuses on the quantitative estimation of proteins, carbohydrates, and lipids in the ethanol extract, mother tincture (Φ), and ultrahigh dilutions (3X, 12C, and 30C) of *Asterias rubens*. This analysis aims to provide a scientific foundation for understanding the biochemical variations across ultra-high dilutions and to support the therapeutic claims associated with this *Asteria rubens*.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Sample

Asterias rubens (common starfish) was collected from the harbour beach (8.7561° N, 78.1790° E) in Tuticorin, Tamil Nadu, India. The collected specimen was washed thoroughly with seawater followed by distilled water to remove sand, debris, and epiphytes. Identification of the species was done using the cytochrome c oxidase subunit I (COI) gene sequencing, followed by sequence similarity search using Basic Local Alignment Search Tool (BLAST) to the GenBank database.

2.2 Preparation of Ethanol Extract and Mother Tincture (Φ)

The cleaned *Asteria rubens* specimen was shade-dried at room temperature and pulverized into a coarse powder. Measured a moisture content (40 ml) by using water bath. The normal biological extraction of *Asteria rubens* (solid - 44.365 gm, strong alcohol - 250 ml) was prepared by Soxhlet apparatus in 12 cycles. As per the Homoeopathic Pharmacopoeia of India, volume.4, for preparing 1 litre of mother tincture (Hahnemann method, class - IV), added 100 gm of pulverized *Asteria rubens* (solid), moisture 360 ml and strong alcohol 637 ml. The powdered material was macerated with strong alcohol for 15 days with intermittent shaking. The percolate obtained was filtered and concentrated to obtain the mother tincture (Φ). After decantation, 870 ml of mother tincture(Φ) was collected, because of absorption of menstruum by the drug material, minor evaporation during processing, and unavoidable filtration and decantation losses and which was stored in airtight amber-coloured bottles for further analysis.

2.3 Preparation of Ultrahigh Dilutions (3X, 12C, and 30C)

Ultra-high dilutions were prepared according to the procedure described in Volume IV of the Homoeopathic Pharmacopoeia of India (HPI).

3X potency (10^{-3}): Drug strength -1/10. One part (10 ml) of the 2X potency was mixed with nine parts (90 ml) of dispensing alcohol was transferred into a fresh amber glass vial. The vial was tightly stoppered. The mixture was subjected to 10 firm succussions by electric potentizer to obtain 100 ml *Asterias rubens* 3X. The preparation was labelled and stored in an amber-coloured glass bottle protected from heat and light.

12C Potency (10^{-24}): Drug strength is -1/100. One part (1 ml) of the prepared 11C, and 99 parts (99 ml) of dispensing alcohol was transferred into a fresh amber glass vial. The vial was tightly stoppered. The mixture was subjected to 10 firm downward succussions by electric potentizer to obtain 100 ml *Asterias rubens* 12C. All succeeding potencies from 13C to 29C are prepared under this scale by mixing one part of the preceding potency and 99 parts of dispensing alcohol.

30C Potency (10^{-69}): Drug strength is -1/100. One part (1 ml) of the prepared 29C, and 99 parts (99 ml) of dispensing alcohol was transferred into a fresh amber glass vial. The vial was tightly stoppered. The mixture was subjected to 10 firm downward succussions by electric potentizer to obtain 100 ml *Asterias rubens* 30C.

All potencies were prepared under aseptic laboratory conditions, transferred into sterile amber-coloured glass bottles, appropriately labelled, and stored at room temperature protected from direct light until further analysis.

2.4 Quantitative Phytochemical Analysis

Quantitative estimation of phytochemical constituents such as alkaloids, flavonoids, phenols, steroids, saponins, and tannins was carried out using standard analytical procedures. The absorbance was measured using a UV-Visible spectrophotometer, and the concentration of phytochemicals was calculated using standard calibration curves.

a) Quantitative Estimation of Proteins

Protein content was estimated by the method of Lowry's method. 1 ml of sample was mixed with 0.5 ml of 0.1 N sodium hydroxide and 5 ml of alkaline copper reagent. The mixture was incubated in room temperature for 30 minutes. Then 0.5 ml of Folin-Ciocalteu reagent was added and incubated again for 10 minutes at room temperature. The absorbance was read at 660 nm against a reagent blank. BSA (bovine serum albumin) used as a standard material and compared the assay with BSA equivalents.⁽¹²⁾

b) Quantitative Estimation of Carbohydrates:

The total sugar content was estimated by Anthrone method (Roe, 1955). A known amount of the sample was taken, ground well with 80% ethanol and was centrifuged at 4000 rpm. From the supernatant, 0.5 ml was taken and 5 ml of anthrone reagent was added. The tubes were kept in a boiling water bath for 15 min. After that, they were kept in a dark room for another 15 minutes. The colour intensity developed was read in a spectrophotometer at 650 nm.⁽¹³⁾

c) Estimation of total lipids

A volume of 0.2 ml of sample was withdrawn and 0.2 ml of concentrated sulphuric acid was added. The tubes were boiled in water for 10 minutes and cooled in cold water for 5 minutes. Upon cooling, 10 ml of phosphovanillin reagent was added. The content was shaken well and incubated at 37°C in a water bath for 15 minutes. The tubes were cooled to room temperature, and the light pinkish brown colour produced was determined at 540 nm after 30 minutes against the reagent blank. A series of standards with varying concentrations from 10-100 µg was used for drawing the standard graph. The lipid concentration of the sample was determined from the graph and was expressed as µg/ml.⁽⁹⁾

3. Results and Discussion

The quantitative estimation of primary phytochemical constituent proteins, carbohydrates, and lipids was carried out for the extract, mother tincture (Φ), and ultrahigh dilutions (3X, 12C, and 30C) of *Asterias rubens* shown in table.1. The results clearly demonstrate variations in biochemical composition across different preparations, reflecting the influence of extraction and potentization processes.

Table 1: Phytochemical Quantitative Analysis

Phytochemical constituent	Extract (µg/ml)	Φ (µg/ml)	3X (µg/ml)	12C (µg/ml)	30C (µg/ml)
Protein	128.96 ± 0.332	104.47 ± 0.009	178.88 ± 0.023	169.48 ± 0.028	138.85 ± 0.012
Carbohydrate	63.87 ± 0.014	42.84 ± 0.016	27.67 ± 0.004	25.86 ± 0.016	27.79 ± 0.077
Lipid	4161.58 ± 0.783	270.25 ± 0.037	140.81 ± 0.004	120.67 ± 0.004	100.41 ± 0.008

3.1 Protein Content

Highest levels of protein were observed in 3X potency (178.88 ± 0.023 µg/ml), followed by 12C (169.48 ± 0.028 µg/ml) and 30C (138.85 ± 0.012 µg/ml). Lower protein contents were recorded in the case of extract (128.96 ± 0.332 µg/ml) and mother tincture (Φ) (104.47 ± 0.009 µg/ml). High levels of proteins in low potencies may be due to good solubilization and dispersion of proteinaceous materials during potentization process.^(14,15) Protein or peptide fractions isolated from marine organisms are known for their anti-oxidative, antimicrobial and anticancer activities.⁽¹¹⁾ The progressive decrease in protein concentration with increase in potency indicates the effect of dilution; nevertheless, the observation of protein even in higher potencies implies the retention of molecular imprints or structural imprints in the potentization process.^(15,16)

3.2 Carbohydrate Content

There was a variation in the carbohydrate contents in the different samples analysed. Ethanol extract had the highest carbohydrate content (63.87 ± 0.014 µg/ml) while mother tincture (Φ) had the second highest carbohydrate content (42.84 ± 0.016 µg/ml). Potency 30C had similar carbohydrate content to potency 3X (27.79 ± 0.077 µg/ml, 27.67 ± 0.004 µg/ml respectively). On the other hand, the lowest carbohydrate content was seen in potency 12C (25.86 ± 0.016 µg/ml). Carbohydrate is important in the structure of cells and energy metabolism of marine life and is known to be associated with immunomodulatory and antioxidant activity in glycoproteins and polysaccharides.^(17,18) Higher carbohydrate content in the extract means efficient extraction of the carbohydrates while stable carbohydrate contents in the potencies show retention of carbohydrate related molecular properties during serial dilutions.^(14,15)

3.3 Lipid Content

A markedly high lipid concentration was observed in extract (4161.58 ± 0.783 µg/ml) compared to all other samples. The mother tincture(Φ) showed a lipid content of 270.25 ± 0.037 µg/ml, whereas the potencies exhibited progressively decreasing lipid levels: 3X (140.81 ± 0.004 µg/ml), 12C (120.67 ± 0.004 µg/ml), and 30C (100.41 ± 0.008 µg/ml). The massive lipid concentration found in the "Extract" highlights *Asterias rubens* as a potent source of marine oils. The lipid content of the starfish is primarily composed of omega-3 fatty acids, a large fraction of these fatty acids is bound to phospholipids rather than triglycerides. Phospholipid-bound omega-3s are theorized to have superior bioavailability because they can integrate directly into cellular membranes.⁽²⁾ The persistence of lipids in the 30C potency suggests that these bioactive phospholipids remain present as structural elements of the preparation's nano colloidal phase. Marine organisms, particularly starfish, are well known for their richness in lipids and fatty acids, which contribute to membrane integrity and biological activity, including antimicrobial and anticancer effects.^(11,19) The exceptionally high lipid content in the ethanol extract confirms the lipid-rich nature of *Asteria rubens*. This trend of reduction in the concentration of lipids with increased potency is expected on account of serial dilution, but the fact of having detectable amount of lipids in higher potencies proves the theory of molecular persistency in ultra-high dilutions.^(14,15)

The presence of proteins, lipids and carbohydrates in 30C dilutions, which amounts to a factor of dilution, is absolutely impossible according to laws of mass action and the Avogadro limit.⁽⁶⁾ The high shear stresses applied to the bulk sample

of starfish during liquid succession causes breakdown of the bulk sample into nano meter size particles.⁽⁷⁾ Nanoparticles of the original material have been found to remain in homeopathic ultra-high dilutions up to 30C and 200C through Transmission Electron Microscopy (TEM) and High Resolution TEM (HRTEM).⁽¹⁾ In the case of biological substances like *Asterias rubens*, the nanoparticles would contain protein, lipids and steroidal glycosides (saponins) complexed with the solvent molecules.⁽¹¹⁾

From the quantitative analysis of the phytochemicals, *Asterias rubens* appears to be a good source of essential biochemical substances, especially lipids and proteins⁽²⁰⁻²⁵⁾. It was found that ethanol extract had high amounts of carbohydrates and lipids while lower homeopathic potencies had higher amounts of proteins⁽²⁶⁻³¹⁾. Biochemical substances are directly linked with the antioxidative, antimicrobial and anti-cancer effects found in the subsequent biological studies.^(11,19) The differences in the amounts of phytochemical substances in extracts, mother tincture and potencies demonstrate the effect of different modes of extraction and potentization on the chemical constitution.^(14,15)

However, the findings shown in Table 1 are consistent with the latest studies which indicate that ultra-high dilutions are not simple solutions but complex colloidal systems of nanoparticles of initial substances.⁽¹⁾ The results provide scientific support for the therapeutic potential of *Asteria rubens* and validate its traditional use in homeopathy.^(1,3)

4. CONCLUSION

The current research represents the quantitative analysis of the principal biochemical compounds-proteins, carbohydrates, and lipids in extract, mother tincture(Φ) and homeopathic ultra-high dilutions of *Asterias rubens* (3X, 12C, and 30C). This research proves that *Asteria rubens* is a source of important biochemical components, namely lipids and proteins, underlining its importance as a medicinal organism from the marine world. Ethanol extract showed the highest content of lipids and carbohydrates due to effective extraction of bioactive compounds. On the contrary, lower dilutions contained higher protein content, and with the increase of the potency, the content of carbohydrates and lipids gradually decreased. At the same time, despite serial dilution, significant number of biochemical components were revealed even in higher potencies, which means that there were some molecular properties remaining after the potentization process. The findings validate the traditional medical application of *Asterias rubens* in homeopathy from the scientific point of view, and serve as a basis for chemical studies regarding the antioxidant, antimicrobial, and anticancer properties of this species.

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