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EFFECT OF ETHANOLIC SEED EXTRACT OF CITRUS LANATUS (WATERMELON) ON IMMUNOREACTIVITY OF HIPPOCAMPAL NEUROFILAMENT OF ADULT WISTAR RATS

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ABSTRACT

Neurofilaments are major protein constituents of the brain, and play important roles in trafficking of molecules between neurons. An experimental study on thetotal protein content and immunoreactivity of neurofilament protein was undertaken on the hippocampus of adult rats treated with the seed extract of *Citrulluslanatus,* Omega H3 and soybean oil. Twenty (20) adult wistar rats of average weights 150g were used. The rats were randomly divided into 5 groups of 4 rats per group. Group A served as control, groups B and C received 100mg/kg and 200mg/kg of *Citrullus lanatus* ethanolic seed extracts orally respectively, while groups D and E received 300 mg/kg of Omega H3 and 5ml/kgof Soybean oil for two weeks respectively. Total serum protein was estimated biochemically and statistically analyzed using SPSS version 20 group comparison were made by one-way analysis of variance (ANOVA) and $P< 0.05$ was considered statistically significant. The hippocampus was dissected, fixed, processed and stained for neurofilament by the avidin–biotin immunoperoxidase method. The result revealed total serum protein levels increased significantly in treated groups while immunoreactivity to neurofilament increased in all treated groups compared to the control. In conclusion, ethanolic seed extract of *Citrullus lanatus* has similar effects as Omega H3 and Soya bean oil which justify it use as supplement.

KEYWORDS: CitrullusLanatus, Cognitive, Neurochemistry, Nutrition, Hippocampus

The use of herbs as nutritional supplements dates back to the time of the early man (1). Thepractice has gained popularity amongst the low income earners in the developing countries including Nigeria. In recent times, daily nutritional intakes can scarcely provide the recommended dietary allowance (RDA), that is the average daily level of intake sufficient to meet the nutrient requirements of nearly all (97%-98%) healthy people. Food and drug supplementations become necessary to make up for a balanced diet. Beside the aforementioned plant supplement: the watermelon also known as *Citrullus lanatus* and a member of the cucumber (*Cucurbitaceae*) family is a popular fruit in many parts of the world and notable for its high water content and attractive look (2).

The watermelon fruit contains numerous seeds which are rich in polyunsaturated fats, vitamins, antioxidants, minerals, proteins in addition to the various phytochemicals which are vital for bodily functions (3). Polyunsaturated fatscontainomega -3 fatty acids which promote brain health and cognitive functions and use source of oil for cosmetic products (2) and plays significant role in the treatment of diseases such as; urinary tract infections, diabetes, hypertension and scabies (3-4).

Like the watermelon seeds, vegetable seed oils such as soybean oil is also rich in polyunsaturated fat. Soybean oil is a popular and most widely consumed cooking oil extracted from the soybean seeds. The soybean seeds are prominent for their high protein and oil content such as monounsaturated fatty acid which is believed to be beneficial in the absorption of vital nutrients and enhancement of cognitive functions of the brain(5-8).

On the other hand, Omega H3is a drug supplement that provides well-balanced source of vital nutrients which can easily be lacking in the diet of many. It contains biologically active, health-promoting important nutritional factors accurately balanced and easily absorbed by the body(9). Omega H3 is rich source of fatty acids which are well characterized by high contents of polyunsaturated fatty acids. Hence, it is used as medicine and nutritional supplement aimed at increasing brain energy and cognitive functions (10).

Food consumption is an intrinsically motivated behavior with the potential to modulate brain structure and function. It has been an established fact that the brain structure is rich in lipids particularly fatty acids(11).

Food and dietary supplements containing high content of polyunsaturated fatty acids are known to enhance brain cognitive function and structural integrity. Particularly, areas of the brain involve in cognition such as the cerebrum and hippocampus. These intakes can mobilize raw materials that can be used to strengthen the cytoskeletal integrity in health and diseases. Thus, this study investigated the effects of ethanolic extract of watermelon seed on immunoreactivity of neurofilament on the hippocampus and, compared with that of soybean oil and omega H3. Since, Neurofilaments are intermediate filaments of matured neurons cytoskeleton and among the most abundant proteins in brain (12-13) and maintains the structural integrity of the brain by transporting proteins and neurotransmitters throughout the neuron. The neurofilament (NF) immunostaining has been used as a common tool in diagnostic neuropathology and differentiating neurons that are positive for NF from glia which shown negative staining for NF.

2. MATERIALS AND METHODS

2.1 Collection and preparation of plant material

Fresh and richly green matured watermelon fruits which were examined to be free of any form of pest bites, damage and were obtained from the local market in Abakaliki, Ebonyi State, Nigeria in the month of August, 2017. It was identified by Prof. (Mrs.) M. O. Nwosu of the Department of Plant Science and Biotechnology, University of Nigeria Nsukka. The fresh watermelon fruits were cut open and the seeds were harvested and allowed to air-dry for five days. The watermelon seeds were grounded into fine powder and used for the preparation of the ethanolic extract. The powdered watermelon seed was thoroughly sieved and 250g of the fine powder was submerged in 500mls of 95% ethanol. The mixture was vigorously stirred and allowed to stand for 48hrs. Thereafter, it was filtered by muslin cloth and the filtrate was left to evaporate for 48hrs. The ethanolic extract was then stored in a bottle and preserved in the refrigerator at 4°c for the administration.

2.2 Drugs and Chemicals

Omega H3 capsules (Vitabiotics Ltd, 1 Apsley way. London. NW27HF (England) was purchased from registered pharmacy store in Abakaliki, Ebonyi State. Soybean oil (Grand oil Nig. Ltd. Nigeria) was procured from the opened market in Abakaliki, Ebonyi State. Routine histological reagents of standard and analytical grades were purchased from certified chemical stores. The neurofilament antibody used was manufactured by Novocastra, LEICA. Germany. The doses of drug were selected based on data from literature and drug information leaflet.

2.3 Experimental Animals / Ethical Statement

Twenty-adult wistar rats of both sexes weighting between 120-150g were purchased from the animal house of Department of Anatomy, Faculty Basic Medicine, Ebonyi State University Abakaliki, Nigeria. The rats were randomly assigned to five groups; A, B, C, D and E, the animals were kept for a period of 2 weeks to acclimatize during this period, they were maintained at room temperature in a ventilated cage and were allowed free access to vital growers mesh and water. All protocols used were in accordance with the ethics and research guidelines of the Faculty of Basic Medical Sciences, Ebonyi State University. After two weeks of acclimatization, the control (group A) was kept on animal feed, groups B and C received 100mg/kg and 200mg/kg of the ethanolic extract respectively. Watermelon seed has the oral median dose (LD_{50}) is 765 mg/kg (14). Groups D and E were administered with 300mg/kg of Omega H-3 and 5ml/kg of soybean oil daily by orally gavages for 2 weeks.

2.3 Termination of experiment

On the day one post administration the rats in all the groups were bledvia the retro-orbital plexususing capillary tubes and the blood samples were collected into a plain sample tubes. Thereafter, the rats were anesthetized using 50mg/kg/bw of thiopentalsodium (Rotexmedica. Trittau. Germany), the transcardiac perfusion wasthen carriedout using 4% paraformaldehyde. The skulls were opened to harvest the brains which were further fixed in 10 % neutral buffered formal saline for 48hours. The cerebellum was removed first then hippocampus was exposed by dividing each cerebral hemisphere into two asymmetrical parts each having an exposed part of the hippocampus and processed for neurofilament immunohistochemical study.

2.4 Estimation of total serum proteins

Total serum protein was photometrically measured at alkaline pH 7.0, which allow proteins to form a stable complex with Cu^{2+}. Three test tubes, blank, standard and sample were labeled respectively. To the sample tube, 0.02ml of serum was added, to the standard test tube, 0.02ml of protein standard was added while; 0.02ml of water was added to the blank test tube. One milliliter (1ml) of the protein reagent was added to the test tubes each. This was mixed well and allowed to stand for 25mins at room temperature (20-25°C). The absorbance was taken at 540nm and calculated thus:Total serum Proteins (in g/dl) = $\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} / \text{g of protein/dl} \times 5$

2.5Immunohistochemical study

Fixed specimens were dehydrated in ascending grades (50%, 70%, 90%, 95% and 100%) of alcohol (ethanol), cleared in xylene and embedded in paraffin wax. Serial sections of 10µm thick were obtained using model 0325 rotatory microtome by Shandon Scientific Ltd, United

Kingdom. The deparaffinized sections were stained using the Avidin - Biotin immunoperoxidase method. The Neurofilament antibody (Novocastra, LEICA. Germany)dilution factor used was 1:100 dilutions. The sections were placed on the hot plate at 70 degree for at least 1hour. Sections were brought down to water by passing them onto 2 changes of xylene, then 3 changes of descending grades of alcohol and finally to water. Antigen retrieval was performed on the sections by heating them on a citric acid solution of pH 6.0 using the microwave at power 100 for 15minutes.The sections were equilibrated gradually with cool water to displace the hot citric acid for at least 5min for the section to cool.

Peroxidase blocking was done on the sections by simply covering section with 3% hydrogen peroxide (H_2O_2) for 15minutes.Sections were washed with phosphate buffered saline (PBS) and protein blocking was performed using avidin for 15minutes.Sections were washed with PBS and endogenous biotin in tissues was blocked using biotin for 15minutes.After washing with PBS, sections were incubated with the respective diluted primary antibody for neurofilament (Novocastra, LEICA Germany) was diluted 1:100 for 60minutes.Excess antibodies were washed off with PBS and a secondary antibody (LINK) was applied on section for 15minutes.Sections were washed and the (LABEL) which is the horseradish peroxidase (HRP) were applied on the sections for 15minutes. A working DAB solution made up by mixing 1 drop (20microns) of the DAB chromogen to 1ml of the DAB substrate. The working solution was applied on sections after washing off the HRP with PBS for at least 5minutes. The brown reactions begin to appear at this moment especially for a positive target. Excess DAB solution and precipitate were washed off with water. Sections were counterstained with Haematoxylin solution for at least 2minutes and blued briefly. Sections were dehydrated in alcohol, cleared in xylene and mounted in DPX. Images were observed, interpreted and captured using Amscope research microscope (Model 3.2. England)

3. RESULTS

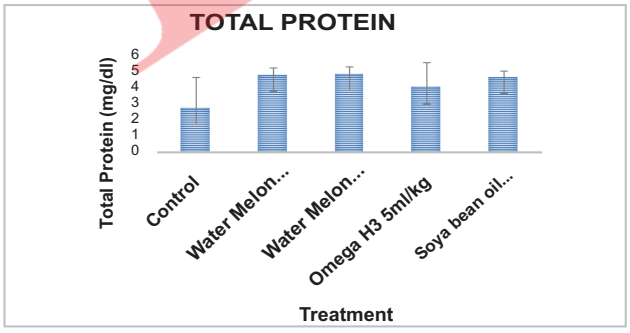
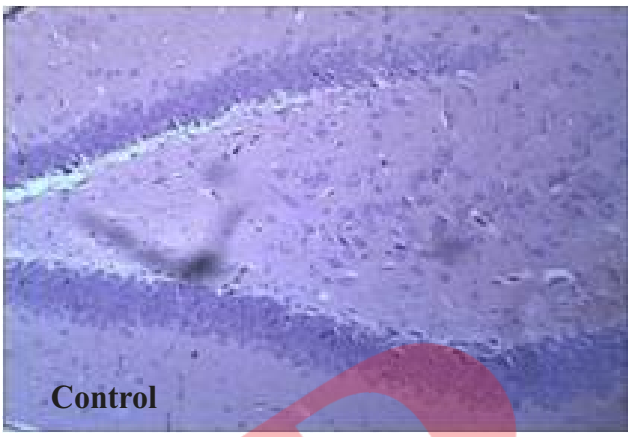
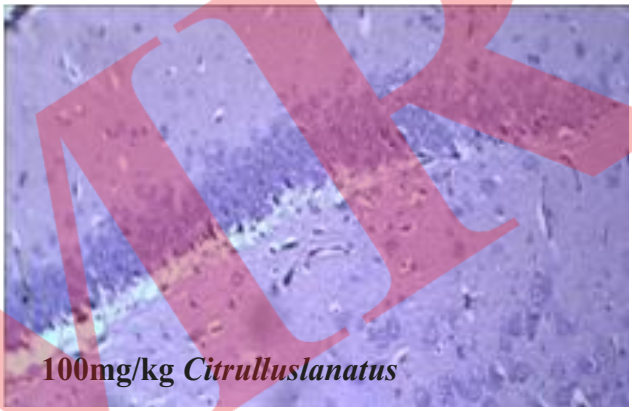
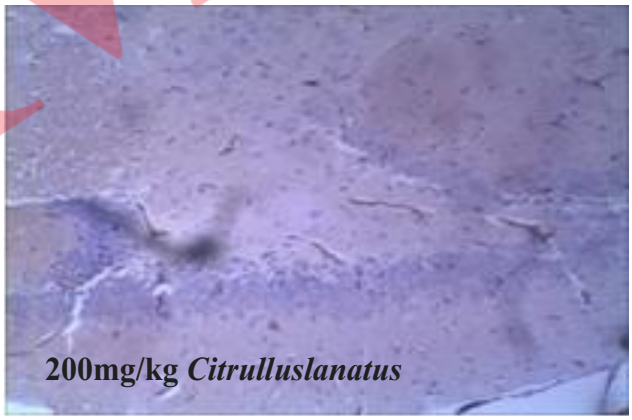
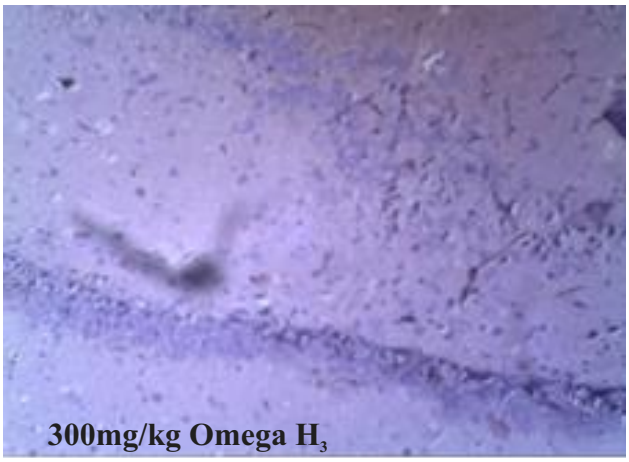


Figure 1: Graph showing the total protein content in each group. Each bar represents mean ± S.E.M. P values for group comparisons were obtained by one way ANOVA followed by Student T- test. P =0.05.









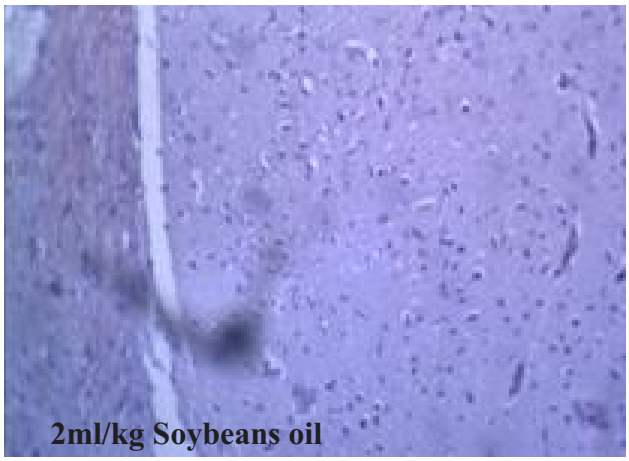


Figure 2:Photomicrograph of the hippocampal tissues of the control,Citrulluslanatus, Omega $H_3$and Soybean oil treated rats. Increase immunoreactivity for neurofilament in all treated groups compared to the control.NeurofilamentImmunostain. x200.

4. DISCUSSION AND CONCLUSION

Our findings revealed that the ethanolic seed extract of *citrullus lanatus*, soybean oil and Omega H_3 significantly increased the total serum protein compared to the control. This affirms that the ethanolic seed extract of *Citrullus lanatus* enhanced the total serum protein. It also comparably increased the total serum protein in a measure similar to the Omega $H_3$and soya beans oil used as standards in the study (figure I).However, no significant difference (p= 0.05) was found to exit between the groups that received 100mg/kg/bw and 200mg/kg/bw of C*itrullus lanatus* ethanolic seed extracts. This implies, the effect of *Citrullus lanatus* on total serum protein is not dose dependent.

Neurofilaments are axonal and synaptic components of neurons. They are known to modulate synaptic neurotransmission and behavior by their differential interaction with specific neurotransmitter receptors in the brain (13).Thence, it plays vital role in cognition while its alterations also underlie axonopathyin neurological disorders and neuropsychiatric diseases (15-18).

The immunohistochemical finding revealed increased immunoreactivity to neurofilament protein antibody in the groups treated with the *Citrullus lanatus* seed extract, Omega H_3 treated group and Soybean oil treated groups compared to the control (figure II). These indicate changes in the cytoskeleton of the hippocampal neurons. Nitin and Milind (3) early reported that the consumption of soybean in diet improves memory and reverses memory deficits. It also showed that *Citrullus lanatus* seed exhibited similar effect as Omega $H_3$and Soybeans in the hippocampus and by implication can enhance cognitive function. Conversely, previous studies confirmed elevated neurofilament reactivity thus improves cognitive function while, decreased neurofilament levels resulted into an impaired cognitive function (19-20).Put together, increased total serum protein and neurofilament will provide an enabling environment for reorganization of the cytoskeleton of neurons thereby strengthens the hippocampal area towards achieving functional homeostasis. This finding can be beneficial in the treatment of disease conditions whereby neuron structural integrity is being compromised as in Alzheimer's disease. In conclusion, the present study shows that *Citrullus lanatus* seed extract has neuroprotective property.

CONFLICTS OF INTEREST

The authors declared that they have no competing interests.

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