Short Research Article

NUTRACEUTICAL POTENTIAL AND ANTIPSYCHOTIC ACTIVITY OF LOCAL DRY FRUIT WASTE – *Juglans regia*

ABSTRACT

Aim : To identify the biologically active components in shells of *Juglans regia* and study its nutraceutical potential and antipsychotic activity for effective waste management.

Study design : Biochemical and *in vivo* analyses of plant extract using established protocols.

Place and duration of study : Sample extraction at Department of Food Science and technology, School of Biotechnology and Bioinformatics, DY Patil deemed to be University, Navi Mumbai, India; sample components identification at Sophisticated Analytical Instrument facility (SAIF), Indian Institute of Technology (IIT), Mumbai, India; and *in vivo* studies for antipsychotic activity using *Caenorhabditis elegans* at Department of Life Science and Biochemistry, St. Xavier's College, Mumbai, India between November 2018 and May 2019.

Methodology : The shells of *Juglans regia* were milled and the extract was prepared using Soxhlet extraction at 60^{o}C using methanol as solvent. The GCMS analysis of the extract was carried out using a GC JEOL – The Accu TOF. Antipsychotic activity was studied using pharyngeal pumping assay in *Caenorhabditis elegans.*

Results : GC-MS analysis of methanolic extract of shells of *Juglans regia* revealed the presence of Tridecanoic acid, Acetoxyacetic acid, nonyl ester, 2-hexenal, 2-ethyl, Eicosanoic acid, phenylmethyl ester, Undecane, Benzeneacetic acid decyl ester, (1-pentyl-allyoxymethoxy-methyl)-benzene), 9,12-octadecadienoic acid(Z,Z), phenylmethyl ester, Benzyl oxytridecanoic acid, 6,9,12- octadecatrienoic acid, phenylmethyl ester (Z,Z), 9- octadecanoic acid (Z), phenylmethyl ester, 9,12,15-octadecatrienoic acid, Z [(trimethyl (sil)oxy, 1 – trimethyl (sily)oxy] ethyl ester (Z,Z,Z). Furthermore, behavioural assay done using *Caenorhabditis elegans* as a model organism showed that the sample exerted antipsychotic activity at lowest concentration.

Conclusion : The shells of *Juglans regia* being a natural source, can be used as an alternative to the synthetic antipsychotic drugs that have side effects. Our current work suggests that the walnut shells that end up into trash bins are an excellent source of effective natural biologically active compounds.

Keywords:

Caenorhabditis elegans, nutraceutical, antipsychotic, walnut shells, gas chromatography mass spectrometry, Pharyngeal pump.

INTRODUCTION:

Edible as well as non-edible parts of plants have been used in the history of mankind for *Ayurvedic* purposes, in food and medicinal drives. Several natural products have been extracted and isolated from various parts of plants for the development of new drugs. There are numerous biological components extracted in their natural forms from medicinal plants, their vegetables and fruits, which possess biological activities. According to research conducted on leaves, bark and kernel of *Juglans regia,* several biologically active components are reported till date. Several natural products, which possess biological activities have been extracted and isolated from various parts of plants for the development of new drugs [12]. One of such natural food sources is dry fruit – English walnut (*Juglans regia*) of family *Juglandaceae*. *Juglans regia* belongs to *Juglandaceae* family and is widely distributed in the northern regions of India [14]. According to *Ayurveda*, several herbal studies in ancient history in Indian traditional medicine represent herbal therapeutics that not only boost memory [13], but also restore cognitive deficits and improves mental function. Components and application of the traditional herb "*Brahmi*" that corresponds to two plants, *Bacopa monnieri* and *Centella asiatica* were studied. A previous study indicates that both plants possess neuroprotective properties and have nootropic activity with therapeutic implications for patients with memory loss [1]. Among dry fruits and berries, study conducted on Goji berries fed to rats decreased anxiety and depression-like behaviours and spatial memory [15]. These properties of goji berries have been attributed to presence of polysaccharides [2]. Psychotic behaviour is a neurological disorder that occurs when an individual suffers from any form of psychosis. The psychotic behaviour could be hallucinations, delusions, drastic change in behaviour, etc. These neurological disorders require research for coming up with antipsychotic drugs for their treatment. Research conducted on several herbs and plants prove their antipsychotic properties. Work on the effect of aqueous stem bark extract of *Amblygonocarpus andongensis* in wistar albino rats showed that the active components of the plant have antipsychotic properties [3]. Non-edible parts of plants too, possess antipsychotic activities; for example: *Albizia zygia* root extracts exhibit antipsychotic-like properties in murine models of schizophrenia. Moreover, a preliminary report of a controlled clinical trial comparing Cannabidiol (CBD) with an atypical antipsychotic drug have confirmed that cannabinoid can be a safe and well-tolerated alternative treatment for schizophrenia as it acts as an antipsychotic agent [4]. To study the effect and mode of action of the drugs produced synthetically or extracted naturally, a nematode – *Caenorhabditis elegans* is used as a model organism. *Caenorhabditis elegans* is an excellent model organism with a well-defined nervous system and is used to model various disease states [16]. In addition, it has a relatively short life cycle and a 3-day generation time from egg to adult stage that can lead to a dramatic increase in the pace of discovery at particularly lower costs of using higher level organism [5]. A previous study has used pharyngeal pumping rate of the organism as an indicator for studying the antipsychotic effect of drugs [6]. Despite availability of drugs for treatment of neurological disorders, they pose a risk of having various side effects. To overcome this problem, drugs can be extracted naturally from several plants having higher biological activity. Thus, herbal extracts can be studied as an alternative for non- traditional antipsychotic drugs. This can be done either by improving the methods of herbal extraction, purification or by enrichment and purification of novel compounds from natural sources to provide novel herbal formulations for

therapeutics in various psychological ailments. As per a report, total waste generated by all the walnut industries in each country is more than 15,000 tonnes/year [7] where most of the shells have no use in most industries except direct combustion in Biofuel industries and Blasting purposes. The research conducted in this study was based on the evidence of research done in ancient times where the shells, bark, leaves and other parts of the plants were used for external as well as internal medicines. In addition to the proven research of all the health-promoting effects of the husk, leaves, bark of all the varieties of walnut, studies were conducted on English walnut (*Juglans regia*) shell as a part of this research. To the best of our knowledge, this is the first report on the evaluation of antipsychotic properties of walnut shells (*Juglans regia*). The objective of the current study was not only to identify the biologically active compounds present in the shells of *Juglans regia*, study antipsychotic activity of the shells of *Juglans regia* and the neurological behaviour, but also to reduce the amount of waste generated by walnut products producing industries for effective waste management. Our finding suggests that the extract of shells of *Juglans regia* contains 12 bioactive compounds namely - Tridecanoic acid, Acetoxyacetic acid, nonyl ester, 2-hexenal, 2-ethyl, Eicosanoic acid, phenylmethyl ester, Undecane, Benzeneacetic acid decyl ester, (1-pentyl-allyoxymethoxy-methyl)-benzene), 9,12-octadecadienoic acid(Z,Z), phenylmethyl ester, Benzyl oxytridecanoic acid, 6,9,12- octadecatrienoic acid, phenylmethyl ester (Z,Z), 9- octadecanoic acid (Z), phenylmethyl ester, 9,12,15-octadecatrienoic acid, Z [(trimethyl (sil)oxy, 1 – trimethyl (sily)oxy] ethyl ester (Z,Z,Z). In addition, Benzeneacetic acid decyl ester [8] of the extract is known to possesses anti-inflammatory activity, which is associated with antipsychotic effects [9], as observed through behavioral assay with *C. elegans*. Whereas Tridecanoic acid [10] and Benzyl oxytridecanoic acid are reported to have antidepressant studies.

MATERIALS AND METHODS:

Collection and processing of plant material: *Juglans regia* shells were collected from APMC market of Navi Mumbai, Maharashtra, India. The decayed shells were separated from the good ones and discarded. The walnut shells were ground into fine pieces and milled in to fine flour using miller.

Extraction of components: 30 gm of the *Juglans regia* (walnut) shell flour was placed in a thimble covered with non-absorbent cotton and was placed in extraction chamber consisting 100 ml HPLC-grade methanol in a Soxhlet apparatus and 200 ml HPLC-grade methanol in the distillation flask. Extraction was carried out at 65^{o} C for 4 hours until the completion of 8 cycles. After completion of extraction, excess of methanol was removed using reflux method. The sample was kept for evaporation at 60^{o} C for 30 h. The extract obtained was in wet powdered form which was reconstituted using 30 ml HPLC-grade methanol (Sigma-Aldrich). The extract was filtered using Whattmann filter paper (Thomas Scientific) to get a clear solution and was stored at -20^{o} C.

Identification of components using GCMS: The GC – MS analysis was carried out using a GC JEOL – The Accu TOF. The ion chamber temperature of the instrument was set to 200^{o}C. Helium was used as a source of inert gas with flow rate of 1 ml/min. The identification of components was based on comparison of their mass spectra with those of NIST Library (Version 12).

***Juglans regia* shell sample preparation and treatment on *Caenorhabditis elegans*:** The methanolic extract obtained was dried at 60^{o}C in order to obtain a powdered form, which was then diluted in 0.1% DMSO. The 35mg/ml stock obtained was further diluted as per 1:100 dilution to get 3.5 mg/ml, 350 µg/ml and 35 µg/ml. Along with sample dilution, vehicle control with 0.1% DMSO (HiMedia) was maintained. The 4 dilutions of the sample prepared were added onto the new nematode growth medium (NGM) plates pre-seeded with *E. coli* OP50. Wild-type adult synchronized *C. elegans* were then transferred to the NGM plates containing the sample and incubated at 25^{o}C for 24 hrs.

Pharyngeal pumping assay: On completion of the 24 hrs exposure time, the worm plates treated with the sample were observed. 2% agar was prepared and pipetted onto clean glass slides. The worms from each of the plates were gently transferred using non sterile M9 buffer to loosen the attached worm from the NGM plates. The worms along with M9 buffer were transferred into 15 ml falcon tubes and allowed to settle using gravity. The supernatant was discarded without disturbing the worm pellet. The worm pellet was loaded onto the agar slides and allowed to dry at room temperature. The slides containing the worms treated with sample were then observed under Tab Microscope at magnification of 10X. Pharyngeal pumping rate was measured under the microscope at 10X for a minute [11].

Statistical analysis:

Data from each group is represented as mean ± SEM. A two-tailed unpaired student's *t*-test was used to analyse statistical differences between sample groups in all the experiments. Level of significance was set at P = 0.05.

RESULTS AND DISCUSSION:

Identification of compounds using GCMS:

Methanolic extracts of shells of *Juglans regia* were prepared using Soxhlet extraction at 60^{o}C. The GC – MS analysis was carried out using a GC JEOL – The Accu TOF (Fig.1). The ion chamber temperature of the instrument was set to 200^{o}C. Inert gas used was Helium with flow rate as 1 ml/min. The compounds identified in the methanolic extract of *Juglans regia* shells are illustrated in Table 1. The compounds Tridecanoic acid and Benzyl oxytridecanoic acid present in the extract of shells of *Juglans regia* are related to antidepressant activities [10]; whereas Benzeneacetic acid decyl ester is related to anti-inflammatory activities associated with antipsychotic activities [9].

Table 1: Compounds Identified in *Juglans regia* shell methanolic extract.

Sr no.	Retention time	Compound
1	4.70 mins	Tridecanoic acid
2	5.43 mins	Acetoxyacetic acid, nonyl ester
3	12.17 mins	2-hexenal, 2-ethyl
4	15.9 mins	Eicosanoic acid, phenylmethyl ester
5	16.28 mins	Undecane

6	21.69 mins	Benzeneacetic acid decyl ester
7	21.90 mins	(1-pentyl-allyoxymethoxy-methyl)-benzene)
8	23.97 mins	9,12-octadecadienoic acid(Z,Z), phenylmethyl ester
9	24.72 mins	Benzyl oxytridecanoic acid
10	25.61 mins	6,9,12- octadecatrienoic acid, phenylmethyl ester (Z,Z)
11	26.62 mins	9- octadecanoic acid (Z), phenylmethyl ester
12	29.54 mins, 35.59 mins	9,12,15- octadecatrienoic acid, Z [(trimethyl (sil)oxy, 1 – trimethyl (sily)oxy] ethyl ester (Z,Z,Z)

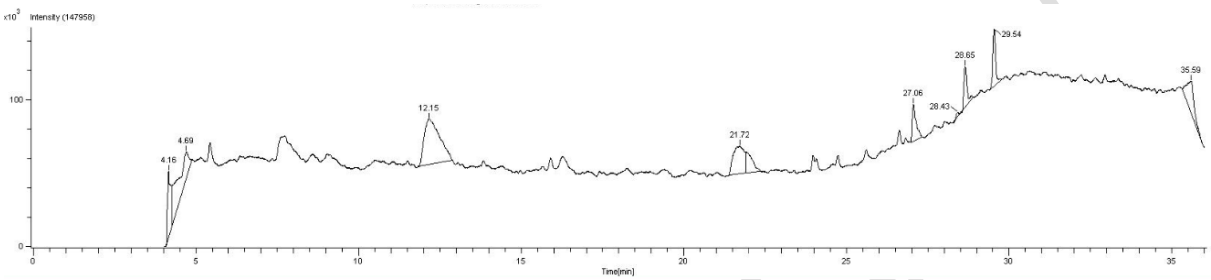

Figure 1: Gas chromatography mass spectrometry graph of compounds identified in methanolic extract of *Juglans regia* shells.

Antipsychotic activity of *Juglans regia* shells on *Caenorhabditis elegans* nervous system:

Pharyngeal pumping assay is preferably used to study antipsychotic drugs in *C. elegans* model system by characterising the molecular and cellular response to these drugs via feeding in the changing behaviour of the organism which is mediated by a well- defined neural circuit. Pharyngeal pumping in *C. elegans* is controlled by pharyngeal nervous system, in particular the MC, M3, and NSM neurons [6]. Following a treatment for 24 hours with a range of sample concentrations (35µg/ml to 35 mg/ml), the worms were tested for pharyngeal pumping capacity. It was observed that with increasing concentration of the extract, the pumping rate increased (See Table 2). However, a two-tailed *t*-test indicated that in all treated worms the rate of pharyngeal pump was significantly lower than that of the vehicle control group (6.53525E-05, 5.86742E-08, 4.11763E-09 and 2.08564E-09 in 35 mg/ml, 3.5 mg/ml, 350 µg/ml and 35µg/ml extract respectively; level of significance was set at P = .05) (Fig.2). This result suggests that some of the components of the extract have a significant antipsychotic effect, which affected the pumping rate in a concentration dependent manner. Pharyngeal pumping rate for different concentrations of the sample are illustrated in the table below:

Table 2: Average pharyngeal pumping min of *C. elegans* after sample exposure for 24 h.

Vehicle control	35mg/ml	3.5mg/ml	350ug/ml	35µg/ml
134	93	64	63	56

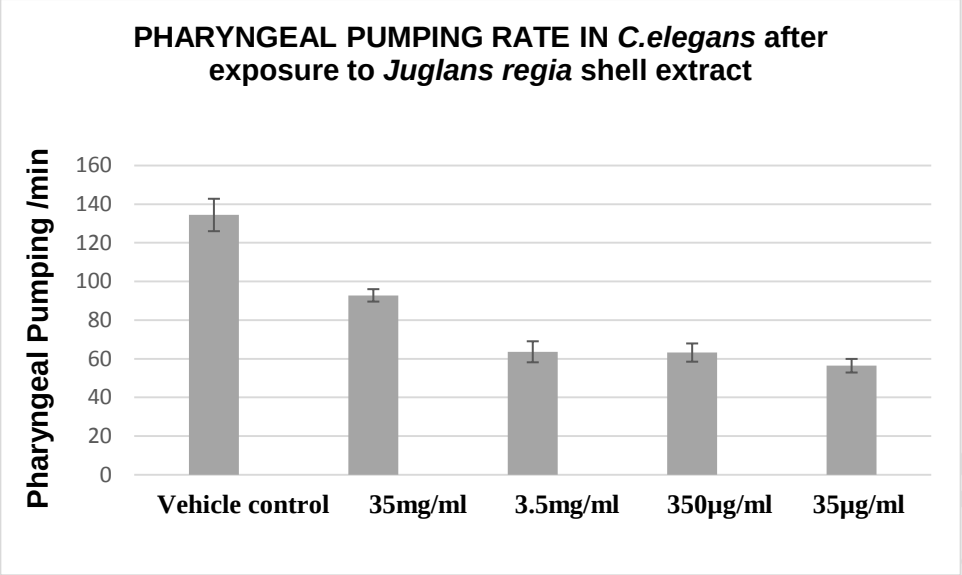

Figure 2 : Average Pharyngeal pumping rate/min of Vehicle control and sample concentrations.

Pharyngeal pumping in *C. elegans* is controlled by pharyngeal nerves, in particular the MC, M3, and NSM neurons[6]. Serotonin stimulates the pumping through a complex response that involves several receptor subtypes, including cholinergic and glutamatergic, but not dopaminergic receptors. *Bacopa monnieri* and *Centella asiatica* plants studies conducted in recent years clearly indicated that both plants possess neuroprotective properties, have nootropic activity with therapeutic implications for patients with memory loss[1]. Similarly, in this study, the pharyngeal pumping rate decreased at a concentration-dependent manner after exposure to the extract of *Juglans regia* shells, indicating that the sample supresses the pharyngeal pumping of the organism that is regulated by the serotonin from NSM neurons. Antipsychotic activity of the extract is attributed to the presence of Benzeneacetic acid decyl ester in the shells of *Juglans regia*. Interestingly, a higher antipsychotic activity of the extract was observed when used at a lower concentration, which can be due to the presence of other compounds such as Tridecanoic acid [10] and Benzyl oxytridecanoic acid that are known to possess antidepressant property. This property of the sample might be due to the effect of the shells of *Juglans regia* on the serotonin secreted from the NSM neurons.

CONCLUSION:

The biologically active components present in the methanolic extract of the shells of *Juglans regia* were identified using a GC JEOL – The Accu TOF, and the components identified were Tridecanoic acid, Acetoxyacetic acid, nonyl ester, 2-hexenal, 2-ethyl, Eicosanoic acid, phenylmethyl ester, Undecane, Benzeneacetic acid decyl ester, (1-pentyl-allyoxymethoxy-methyl)-benzene), 9,12-octadecadienoic acid(Z,Z), phenylmethyl ester, Benzyl oxytridecanoic acid, 6,9,12- octadecatrienoic

acid, phenylmethyl ester (Z,Z), 9- octadecanoic acid (Z), phenylmethyl ester, 9,12,15-octadecatrienoic acid, Z [(trimethyl (sil)oxy, 1 – trimethyl (sily)oxy] ethyl ester (Z,Z,Z). The extract studied showed that the phytochemical constituents have great potential for regulating serotonin from the NSM neurons. Thus, the sample exerted an antipsychotic effect on *Caenorhabditis elegans.* This plant-derived bioactive mixture of the compounds can potentially be used as source of antipsychotic drugs in pharmaceutical industries and on further confirmation by the clinical trials. The extract of shells of *Juglans regia* being a natural source, can be used as an alternative to the synthetic antipsychotic drugs having side effects. Thus, the results obtained in this research indicate that the walnut shells that end up into trash bins consist of important components that are an excellent source of effective natural biologically active compounds.

Ethical: NA
Consent: NA

REFERENCES:

1. Shinomol George and Murali Muralidhara; Exploring the Role of “Brahmi” (Bocopa monnieri and Centella asiatica) in Brain Function and Therapy, Recent Patents on Endocrine Metabolic & Immune Drug Discovery 5(1):3349 · January 2011.
2. Fatma Pehlivan Karakas, Hamit Coskun, Kadir Saglam, Bihter Gokce Bozat, Lycium barbarum L. (goji berry) fruits improve anxiety, depression-like behaviors, and learning performance: the moderating role of sex; Turk J Biol (2016) 40: 762-771.
3. Ebbo Abdullahi Aliyu., Elsa Abdullahi Teleh., Etuk Emmanuel Udok., Ladan Mohammed Jengebe, Saganuwan Alhaji; Antipsychotic effect of aqueous stem bark extract of Amblygonocarpus andongensis in wistar albino rats; Animal Research International (2008) 5(2): 831 – 834.
4. A.W. Zuardi, J.A.S. Crippa, J.E.C. Hallak, F.A. Moreira, F.S. Guimarães; Cannabidiol, a *Cannabis sativa*constituent, as an antipsychotic drug; Braz J Med Biol Res, April 2006, Volume 39(4) 421-429 (Review).
5. Eric A. Engleman, Simon N. Katner, Bethany S. Neal-Beliveau*; Caenorhabditis elegans* as a model to Study the Molecular and Genetic Mechanisms Of Drug Addiction; Progress in Molecular Biology and Translational Science, Volume 137; ISSN 1877-1173.
6. Dallas R. Donohoea,1, Raymond A. Jarvisb, Kathrine Weeksc, Eric J. Aamodtc, Donard S. Dwyer; Behavioural adaptation in C. elegans produced by antipsychotic drugs requires serotonin and is associated with calcium signaling and calcineurin inhibition; Journal Neuroscience Research 64 (2009) 280–289.
7. Rayomand Engineer. 2017. Now, Kashmir’s Discarded Walnut Shells Can Be Used to Make Batteries! Sodium-ion batteries are significantly cheaper than lithium-ion varieties. https://www.thebetterindia.com/125277/kashmir-walnut-shells-can-make-batteries/ visited 09.07.2019

8. Chongxi Yu (2011) U.S. Patent No. US 20110269689A1, Washington, DC: U.S. Patent and Trademark Office. United States Patent Application Publication, Pub. No.: US 2011/0269689 A1, Pub. Date: Nov. 3, 2011. https://patentimages.storage.googleapis.com/b8/8b/b2/92f5ca0c14f657/US20110269689A1.pdf
9. Iris E Sommer; Lot de Witte, Merieke Begemann, Rene S Kahn; Non Steroidal Anti-inflammatory drugs in Schizophrenia : Ready for Practice or a good start ? A Meta – analysis; J Clin Psychiatry 2012; 73 (4) : 414 – 419.
10. Dan Peters, Carl- Emil Sandberg (2002) U.S. Patent No. WO 03/042162 A1, Washington, DC: U.S. Patent and Trademark Office.
11. Elizabeth R Sawin, Rajesh Ranganathan, H.Robert Horvitz, C. elegans Locomotory Rate Is Modulated by the Environment through a Dopaminergic Pathway and by Experience through a Serotonergic Pathway; Cell| Volume 26, Issue 3, Pg 619-631, June 01, 2000. 30.
12. Cragg, G. M., & Newman, D. J. (2013). Natural products: a continuing source of novel drug leads. *Biochimica et Biophysica Acta (BBA)-General Subjects*, *1830*(6), 3670-3695.
13. Pandey, M. M., Rastogi, S., & Rawat, A. K. S. (2013). Indian traditional ayurvedic system of medicine and nutritional supplementation. *Evidence-Based Complementary and Alternative Medicine*, *2013*.
14. Hernández-Alonso, P., Camacho-Barcia, L., Bulló, M., & Salas-Salvadó, J. (2017). Nuts and dried fruits: An update of their beneficial effects on type 2 diabetes. *Nutrients*, *9*(7), 673.
15. KARAKAŞ, F. P., COŞKUN, H., SAĞLAM, K., & BOZAT, B. G. (2016). Lycium barbarum L.(goji berry) fruits improve anxiety, depression-like behaviors, and learning performance: the moderating role of sex. *Turkish Journal of Biology*, *40*(4), 762-771.
16. Raley-Susman, K. M., Chou, E., & Lemoine, H. (2018). Use of the Model Organism Caenorhabditis elegans to Elucidate Neurotoxic and Behavioral Effects of Commercial Fungicides. *Neurotoxins*, 37.