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Effect of commercial Adaptogen preparations of *Withania somnifera, Eleutherococcus senticosus, Echinacea species* on fertility of *Caenorhabditis elegans*

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Abstract
Physiological stress is one major environmental factor that influences fertility and longevity. Stress management has been shown to improve both fertility and longevity in *Caenorhabditis elegans* (*C. elegans*). *C. elegans* is commonly used as an *in vivo* model of human diseases such as stress and used in pharmaceutical research. Adaptogens are plant species that contain phytochemicals that assist the body via the HPA axis and heat shock proteins to manage stress, with species including; Withania (*W. somnifera*), Siberian Ginseng (*Eleutherococcus senticosus*), and Echinacea (*E. purpurea, E. angustifolia*). Stress reduction, fertility and longevity and thought to be inter-connected. In this study commercial extracts of four adaptogens were exposed to wild-type *C. elegans* worms (N2) at dosages suggested for human use and adjusted for the live weight of the worms. The worms were grown at 20 ºC using 'C. elegans Habitation and Reproduction media (CeHR) until the worms reproduced (21 days - L4 stage). Treatment with all adaptogens mentioned significantly improved fecundity compared to control by 186%, 164%, 169% and 205% with *W. somnifera*, *E. senticosus*, with Echinacea Regular, and Echinacea Premium, respectively. A positive association exists between fertility and longevity, and thus these practitioner adaptogenic formulas display potential for fertility and anti-aging therapies with further research required to elucidate the molecular mechanisms by which these changes in fertility and longevity occur.

Keywords: *Caenorhabditis elegans,* Fertility, Longevity, Adaptogen, Echinacea, Withania, Siberian ginseng

1. Introduction
Physiological stress is one of the most common environmental factors that influence the fertility and longevity in *Caenorhabditis elegans* (*C. elegans*). Previous studies have demonstrated an increase in longevity and fecundity of *C. elegans* with adequate stress management or stress resistance [1]. Stress triggers the activation of the hypothalamo-pituitary-adrenal (HPA) axis, which increases the ability to cope with stress through several mechanisms and may adversely affect longevity and fecundity [2, 3]. Adaptogens are specific natural phytochemicals that are helpful in stress management. These adaptogens act as a tonic and assist the body to normalize physiological processes that increase stress [4]. Withania somnifera (*W. somnifera*), *Siberian Ginseng* (*Eleutherococcus senticosus*), and *Echinacea* (*E. purpurea, E. angustifolia*) are some commonly used adaptogens in the management of stress. Wiegant *et al.* demonstrated a dose-dependent improvement in fecundity and longevity with the use of adaptogens in *C. elegans* [5]. The present study aimed to evaluate commercial adaptogen extracts with defined phytochemicals present for their adaptogenic properties related to fertility and longevity using a *C. elegans* model.

2. Materials and Methods
2.1 Worm Strain and Culture
Wild-type *C. elegans* worms (N2) were obtained from *C. elegans* genetic center (CGC) and used for all experiments. Worms were grown on a chemically defined axenic media known as *C. elegans* Habitation and Reproduction (CeHR) media which is composed of 40 chemicals including salts, vitamins and growth factors, nucleic acid, essential and non-essential amino acids buffers and ultra-heat treated organic skim milk. As seen in Figure 3, there is no significant difference between the survival of worms in either CeHR versus liquid NGM media and thus neither influence lifespan in respect to the other. CeHR media is both light and heat sensitive, therefore it is a necessary to wrap the different stock solutions as well as the assembled media before and during culturing the worms, with aluminum foil. Filtering, auto-claving and application with a cocktail of three anti-biotics were included to achieve axenic

culturing media [6]. Replacing the commonly used *Escherichia coli* food used in NGM media with this axenic media has numerous advantages including avoiding the nutrient metabolizing effects of the bacteria of the adaptogens and enables a clear assessment of the direct impacts of treatments (i.e. phytochemical ingestion) on the worms, rather than possible secondary metabolites created within the *E. Coli* when consumed by the worms. Worms were cultured at 20 ˚C for all experiments.

2.2 Fertility and Longevity Assays

The HPLC analysis of *W. somnifera, E. senticosus,* and *Echinacea* (Regular and Premium) was provided by Mediherb (Integria Healthcare, Australia) (Figure 1A, Figure 1B, and Figure 1C). *C. elegans* of Larvae4 (L4) stage were added to the individual wells. The doses of adaptogens to be added in individual wells were calculated based on 5 µg wet weight. The manufacturer of these adaptogens provided the average lifespan in media for 21 days. However, additional extracts were added accordingly, if evaporation occurred. The fertility and longevity assays were conducted on 96 well plates (24 well plates for each adaptogen) with one wild-type *C. elegans* worm per 100 µL CeHR media per well. The effect of each adaptogen was analyzed in six different well plates. The fertility was evaluated in terms of number of offspring and lifespan of *C. elegans* after the addition of adaptogen extracts. The evaluation was performed at regular intervals of time and compared with a control.

2.3 Treatments and Statistical Analysis

The effect of four herbal extracts (*Withania somnifera*, *Eleutherococcus senticosus*, *Echinacea purpure* and *Echinacea angustifolia*) on fertility was compared against the control (CeHR Media) which was the standard culturing media without any herbal extract added. The average, standard error, and t-test were performed using MS Excel 2010 to compare significant effects of the treatments on fertility of the worms.

3. Results

3.1 Effect on fertility of *C. elegans*

Treatment of L4 stage *C. elegans* with various adaptogens resulted in a significantly improved fecundity as compared to control (no adaptogens). The improved fecundity was calculated in terms of the number of hatched offspring after treatment with adaptogens. Treatment of *C. elegans* with *W. somnifera* gave rise to 39 offspring; *E. senticosus* gave rise to 34; *Echinacea* Regular gave rise to 36, and *Echinacea* Premium gave rise to 43 hatched offspring when compared to 21 offspring in the control group (Figure 2). The fertility increased by 186% with *W. somnifera*, 164% with *E. senticosus*, 169% with *Echinacea* Regular, and 205% with *Echinacea* Premium, respectively. The highest increase in fecundity was observed after treatment with *Echinacea* Premium.

4. Discussion

A positive association has been observed between fertility and longevity in *C. elegans*. An extended lifespan has been observed in this nematode after the completion of its reproductive cycle. This extension in lifespan occurs as a result of mutations within this nematode. *C. elegans* has shown decreased lifespan with poor fecundity [7]. Among many environmental influences, stress is known to impact fertility in *C. elegans* to the highest extent. This begins with the activation of parvocellular neurons in the paraventricular nucleus of the hypothalamus (PVN). The HPA axis is activated due to stress. Stress is considered to be an adaptive response that may result in harmful effects including immunosuppression and peripheral muscle dysfunction on a long-term basis [3]. Adaptogens are known to provide relief from stress and enhance energy levels. The present study has shown considerable improvement in fertility with adaptogens including *W. somnifera, E. senticosus*, and *Echinacea* spp in a nematode model using *C. elegans*. Previously conducted studies have also demonstrated improved fecundity with these adaptogens in *C. elegans*. Kumar *et al*. have shown increased longevity in *C. elegans* with the use of *W. somnifera* through various mechanisms [8]. Another study conducted by Wiegant *et al.* showed that the use of *E. senticosus* is beneficial in the reduction of stress and enhancement of longevity. Reduction of stress may lead to improved fecundity with the use of *E. senticosus* in *C. elegans* [5]. From the HPLC analysis of the adaptogens (Figure 2) a number of phytochemicals were elucidated. As seen in this experiment, the commercially available adaptogen extracts increased hatching rate (i.e. fertility) which is linked to longevity. Stress is a causative factor for male infertility. *Withania somnifera* (5g/d) has been documented to increase fertility in normozoospermic but infertile individuals via stress reduction possibly via increased plasma anti-oxidants and semen quality [9], and increases spermatogenic activity using a root extract in oligospermic patients (675 mg/d t.i.d. for 90 days) and resulted in significant increase in sperm count, semen volume and sperm motility [10]. A key phytochemical elucidated in the Siberian ginseng commercial extract was Eleutherosides. In a study in BALB/c mice (in-breed cancer prone strain) taking a mixture of three herbal extracts including *Eleutherococcus senticosus*, augmented endurance of mice to increased time taken to exhaustion in a dose-dependent manner and found to be attributed to increases serum heat shock protein 70 (Hsp70) and Hsp72 (i.e. cellular defence responses to stress) [11] as per Hsp90 in *Drosophila* melanogaster [12]. Moreover, Echinacosides which are phenylethanoids are present in *Echinacea spp.* have also been isolated from the stems of *Cistanches salsa*, reduce tumor necrosis factor-α induced apoptosis in human neuroblastoma cell line; SHSY5Y and thus Echinacosides exerted a neuroprotective effect and thus of benefit for neuro-degenerative diseases in part due to anti-oxidative stress effects, improved mitochondria function, and inhibition of caspase-3 activity (i.e. reduced cleavage of amyloid-beta 4A precursor protein), and increased anti-apoptotic protein Bcl2 [13], and thus having a neuroprotective effect via inhibition of apoptosis. Moreover, caftaric acid also present in *Echinacea spp*., protects sperm synthesis from xenotoxins i.e. synthetic pyrethroids (lambda cyhalothrin), which cause significantly increased oxidative damage to testicles i.e. elevated malondialdehyde, catalase, superoxide dismutase, glutathione-S-transferase activities, and sperm abnormalities and a significant reduction in testicular glutathione concentration, sperm count, sperm motility, and a live sperm percentage [14]. Another component, cichoric acid**,** which is a caffeoyl derivative and acts as a potent reactive oxygen species (ROS) scavenger, and thus oxidative stress conditions via the AMP-activated kinase (AMPK) pathway (cellular substrate homeostasis) and enhanced glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities and thus reduced cellular oxidation [15]. In conclusion, the results of the present study have shown a positive correlation between the use of adaptogens and reduction in physiological stress levels through various physiological mechanisms, which reflected in increased fecundity in wild-type *C. elegans*

model. However, further research in this area may provide stronger evidence for the use of these adaptogens for improving fecundity.

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Conflict of Interest
No conflict of interest or financial incentive for conducting this research.

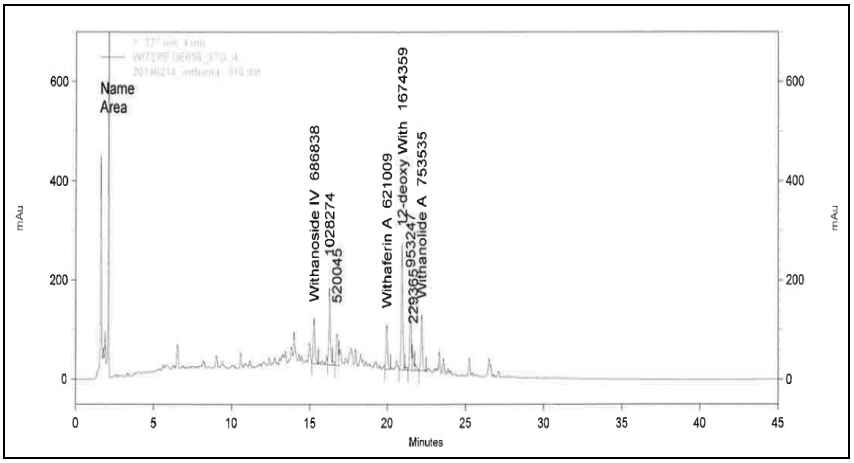

Fig 1A: Active Compounds of *W. somnifera*: HPLC Trace Analysis

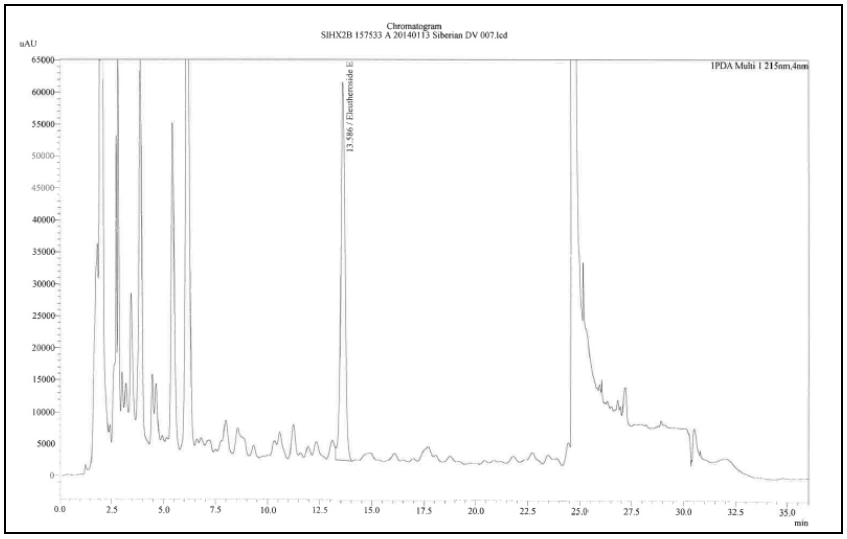

Fig 1B: Active Compounds of *E. senticosus*: HPLC Trace Analysis

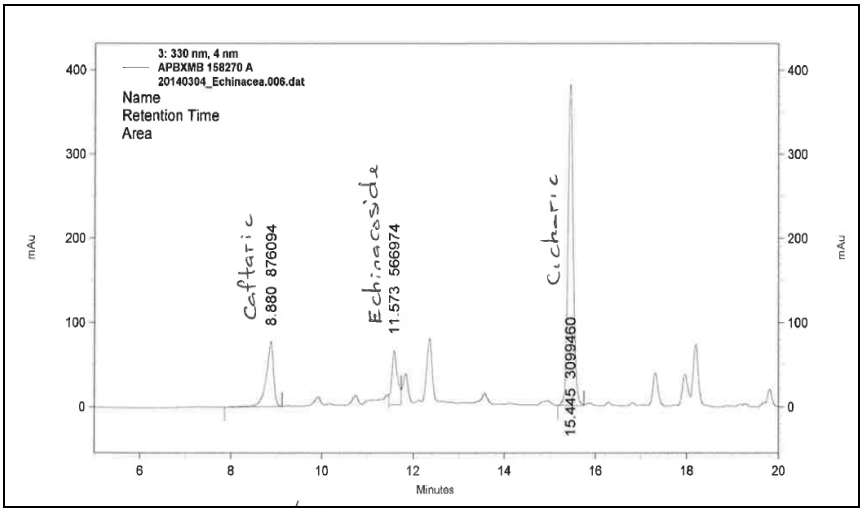

Fig 1C: Active Compounds of *Echinacea*: HPLC Trace Analysis

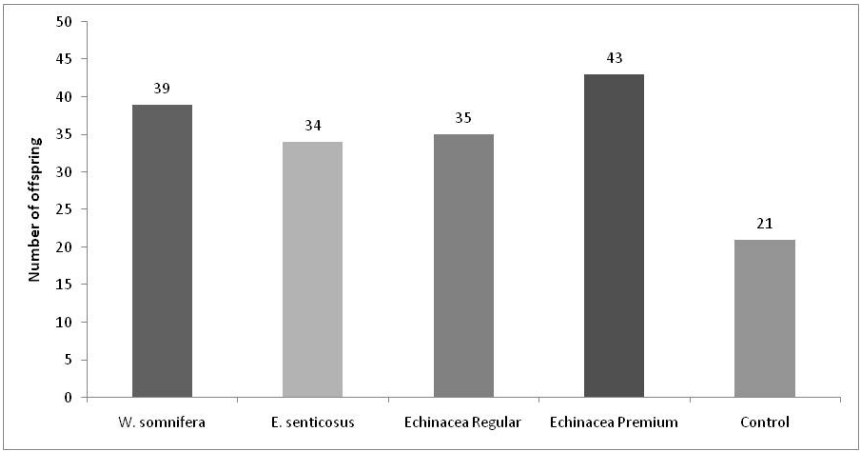

Fig 2: Number of Offspring Resulting after Treatment with Each Adaptogen

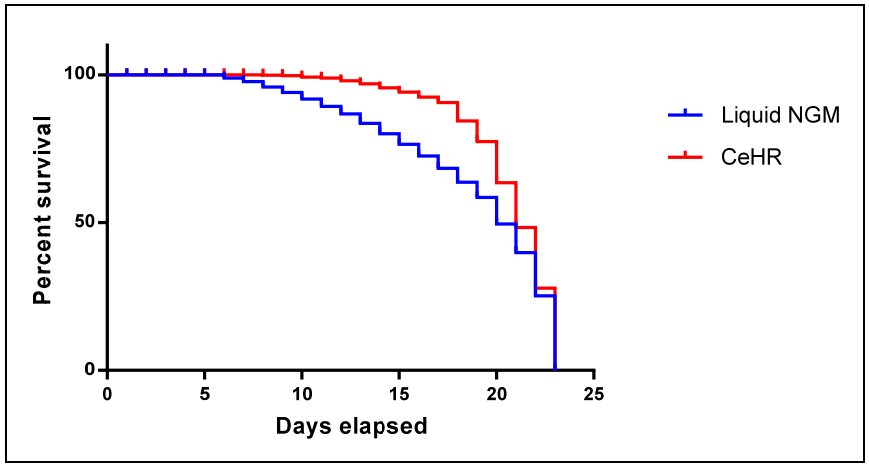

Fig 3: Survival of *C. elegans* worms in defined axenic medium (CeHR) compared to liquid bacterial medium (NGM). Number above bar refers to number of offspring. [All treatments significant $p<0.01$ when compared with control]

There was significant difference in survival of worms in the two media but the median life span (number of days elapsed) was not statistically different.

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