

Effects of withania somnifera root extracts on serotonin secretion in suiz albino mice

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Abstract

Pain is a common manifestation of presence of disease or physical injury in humans. High levels of serotonin in the plasma has been associated with pain. This study aimed at determining the effect of *Withania somnifera* root extracts on serotonin levels in Suiz albino mice. *W. somnifera* root samples were collected and ground to small pieces. Ethanol, n-butanol, xylene and methanol were used to extract metabolites from the ground root materials using hot extraction technique. The mice were injected with the extracts separately and serotonin levels determined over a period of 12 h at 2 h intervals. Serotonin concentration was determined using Beer Lambert's method. Light absorbance by the extracts varied significantly ($F = 50.2011$ $P = 1.25 \times 10^{-07}$). The concentrations of serotonin from mice injected with the metabolites from the selected solvents varied significantly ($F = 23.3269$ $P = 0.0000469$). In addition, there was no significant difference in light absorbance values obtained by Panadol, methanol and ethanol extracts. ($F = 3.7178$ $P = 0.089$). Extracts from *W. somnifera* have the ability to reduce serotonin levels in plasma. Further studies on the mechanisms involved in reducing serotonin levels by *W. somnifera* in blood need to be carried out. There is need for mass production of metabolites from *W. somnifera*.

Keywords: *Withania somnifera*, Root extracts, Serotonin, Suiz albino mice.

Introduction

Serotonin (5-hydroxytryptamine) is a monoamine neurotransmitter. It is a derivative of tryptophan (Kauray et al., 2013). Serotonin is predominantly found in the gastrointestinal tract, platelets and in the central nervous system of animals. Approximately 90% of the total serotonin is located in the enterochromaffin cells in the alimentary canal, where it is used to regulate intestinal movements (Maheswari and Manisha, 2015). The serotonin secreted from the enterochromaffin cells eventually finds its way out of tissues into the blood where it is stored (Rai et al., 2016).

High levels of plasma serotonin have been observed to be in direct proportionality with increase in pain (Abeer et al., 2016). However, since *W. somnifera* extracts have been found to reduce pain, the effect of the plant on the serotonin levels in blood has not yet been established. Reduction of plasma serotonin levels will be taken as an indicator to reduction in pain up to the required standards (Sivamani et al., 2014). Substances that reduce pain will be expected to reduce serotonin levels to the required amounts for positive physiological processes or at least reduction of serotonin levels as a course for pain reduction (Khan et al., 2015).

Withania somnifera (Solanaceae) is a xerophytic plant, found in the drier parts of Kenya, India, Sri Lanka, Afghanistan, Baluchistan and Sind and is distributed in the Mediterranean regions where it grows wild on waste places and on road sides (Umadevi et al., 2016). It is also cultivated for medicinal purposes in fields and open grounds throughout India but not much research has been done in Kenya. The chemistry of *W. somnifera* has been extensively studied and over thirty-five chemical constituents have been identified, extracted, and isolated (Giri, 2013). The biologically active chemical constituents are alkaloids (isopelletierine, anaferine), steroidal lactones (withanolides,

withaferins), saponins containing an additional acyl group (sitoindoside VII and VIII), and withanolides with a glucose at carbon 27 (sitoindoside IX and X) (Wadhwa et al., 2015). *W. somnifera* is also rich in iron. Research conducted at the Department of Pharmacology, University of Texas Health Science Center indicated that extracts of *Ashwagandha* produce GABA-like activity, which may account for the herb's anti-anxiety effects (Durg et al., 2016). The chemopreventive effect was demonstrated in a study of *ashwagandha* root extract on induced skin cancer in Swiss albino mice given *ashwagandha* before and during exposure to the skin cancer-causing agent (7,12-dimethylbenz (a) anthracene) (Kedia and Chattarji, 2018).

W. somnifera - Dunal (locally known as *Ashwagandha*) is widely used in Ayurvedic medicine, the traditional medical system of India. It is an ingredient in many formulations prescribed for a variety of conditions such as arthritis and rheumatism (Jayanthi et al., 2014). The plant has also been utilized as a general tonic to increase energy, improve overall health and longevity, and prevent disease in athletes, the elderly, and during pregnancy (Nabeel et al., 2013). It has been reported that *W. somnifera* exerts significant effect on the endocrine system. Based on the observations, that *W. somnifera* provides protection from free radical damage in the mouse liver; studies were conducted to determine the efficacy of *W. somnifera* in regulating thyroid function (Vidvashankar et al., 2014). The treatment significantly increased the serum levels of 3, 3'-5-tri-iodothyronine (T3) and tetra-iodothyronine (T4), while the hepatic concentrations of glucose 6-phosphatase activity and hepatic iodothyronine 5'-monode-iodinase activity did not change significantly (Alam et al., 2015).

Back pain and arthritis are increasingly becoming a complex clinical challenge in the present society. According to the World Health Organization (WHO), up to 60% of the

human population suffers from these complications at some stage in their life time (Bharathi et al., 2015). Arthritis is characterized by the wearing of joints' soft bone, caused by failure of osteopoline, a protein responsible for calcium deposition in the osteoclasts of the bone and cartilage. This condition predisposes an individual to other clinical disorders thus impacting negatively on their general health status (Khan et al., 2015). To address pain caused by musculo-skeletal disorders and providing a permanent solution has been a great challenge over generations. The present clinical remedies to these pains give a short lived relieve and therefore treatment with a longer lasting relieve from pain or total pain reduction is lacking. *W. somnifera*, being a potent pain modulator would probably modulate serotonin levels in the blood stream thereby reducing joint and back pains. This study aimed at determining serotonin levels in Swizz albino mice injected with root extracts of *W. somnifera* using xylene, methanol, ethanol, normal butanol and water as solvents.

Materials and methods

Study area

The study was conducted at Egerton University, main campus Njoro in Kenya. Egerton University is located in Njoro sub county with coordinates as 0° 23' south, 35° 35' and an altitude of 200m above sea level. Temperature range between 17-22°C While the average annual rainfall is 100mm (Amata et al., 2014).

Sample collection and preparation

W. somnifera root samples were collected from Baringo County, around Chemeron Campus, Egerton University around noon when physiological processes of the plants were expected to be optimal. Approximately 10 healthy plants were uprooted by digging the surrounding earth to reach the tip of the taproots. A pair of secateurs was used to chop the plant borders and separate roots from stems. The root samples were packed in sterile plastic bags and transported to the Department of Biological Sciences laboratories Egerton University, Njoro. The samples were dried in the shade for 1 month. Extraction of crude extracts using selected organic solvents was carried out serially (Amitabha et al., 2016).

Preparation of the solvents

The following solvents were prepared and used as guided by Abeer et al. (2016).

1. 1.171g of ethanol extracts which was dissolved to make 20 milliliters with distilled water (0.05855g/ml).
2. 1.027g of n-butanol extracts that was dissolved to make 20 milliliters solution with distilled water (0.05135g/ml).
3. 1.256g of xylene extracts which was dissolved to make 20 milliliters with distilled water (0.0628g/ml).
4. 1.082 g of methanol extracts that was dissolved to make 20 milliliters solution with distilled water (0.0541g/ml).

Extraction of crude extracts

Extraction of crude extracts was carried out using hot extraction technique (Sivamani et al., 2014). Five hundred grams dry plant roots were ground to powder form and placed in a Winchester bottle containing 1.5 liters of distilled methanol. Incubation at room temperature (23±2°C for 24 h was carried out followed by filtration using Whitman number 1 filter papers. Distilled methanol was repeatedly added to the residue until it became colorless each washing being allowed 24 h before filtration. The filtrate was transferred into a round bottomed flask which fitted into the rotary evaporator. A second round bottomed flask was fitted on the opposite side to the first so as to collect the solvent being cooled (Tanna et al., 2014).

A fraction of methanol crude extract was transferred into a separating funnel up to about three-quarters full then to five-sixths full with methanol and thoroughly shaken. The water based extracts were drained into a beaker while ethanol based ones were put into a new Winchester bottle. The polar extracts were washed with n-butanol in the separating funnel until final washing n-butanol was colorless as above, adding every fraction to the Winchester bottle. This resulted in n-butanol-phase polar extract and methanol phase polar extract. This was repeated with xylene and ethanol.

Withania somnifera extract bioassay with mice

Selection and preparation of mice

Five sets of four healthy males and mature albino mice (Fig.1) were obtained from The Department of Biological Sciences, Egerton University. The selected mice were kept in quiet and spacious cages to acclimatize while being fed ad libitum with standard mice pencils (which are in form of pellets) and water (The Prevention of Cruelty to Animals Act chapter 59 of 1960). The first set of 4 mice was then injected with 2 ml of *W. somnifera* root extract methanol fraction. This was repeated using ethanol, n-butanol, water, and xylene extracts. Blood was harvested according to Khan et al. (2015). Blood serotonin levels was determined over a period of 12 h at 2 h intervals, recorded and compared for the five sets. The sixth set was injected with 2ml of *W. somnifera* extract that had been boiled for 10 minutes from the best performing extract fraction. Serotonin levels were taken at intervals of 2 hours for 5 times after every 2 hours and recorded. Blood serotonin levels were determined at 2 hourly intervals for 12 hours and recorded. The negative control set was had blood serotonin levels determined without any treatment with the extracts. For the positive control experiment, Panadol was used as a non-steroidal anti-inflammatory drug (Kauray et al., 2013).



Fig. 1: Selected mice

Determination of serotonin levels

Platelet-rich plasma was prepared from 1 to 1.5 mL of blood from each mouse (Kamal et al., 2017). The platelets were spun down at 1600g for 20 minutes at 0 to 4°C. The supernatant was discarded and the platelets stored in a deep-freezer. Distilled water up to 1 ml. was added to the button of platelets, and thoroughly suspended. Then 0.2ml of a 10% solution of ZnSO₄.7 H₂O was added, followed, after briefly shaking, by 0.1 ml of 1 M NaOH. After vigorous shaking for one minute, the test-tubes were centrifuged at 1600 g for 15 minutes and then 0.5 ml of the water-clear supernatant was put in a test-tube, 0.15ml of concentrated HCl added, and serotonin fluorescence checked at 295 and 540 nm using a spectrophotometer. A blank (1 ml of distilled water) and one or more standards (1 ml of a 0.5µg/ml solution of 5HT base) was run throughout the procedure.

Determination of serotonin concentration

The mean concentrations of serotonin from mice treated with Withania somnifera was determined using Beer Lamberts equation (Alam et al., 2015);

$$A = \sum mcl$$

Relationship between absorbance and concentration of the metabolites

The y-intercept of the best line of fit was at 0.2 (Fig. 2). Absorbance values at 0.6, 1.7, 1.8, 2.2 and 2.4 were the same with concentration values of 0.07, 0.23, 0.24, 0.33 and 0.37 respectively.

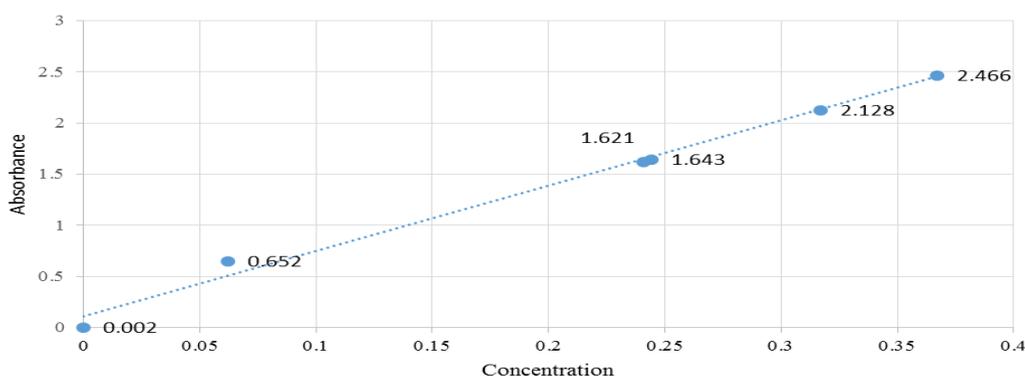


Fig. 2: Relationship between absorbance and concentration of serotonin from mice treated with different cold extracts of W. somnifera roots

where; A = Absorbance, $\sum m$ = Molar extinction coefficient (6718), C = Concentration and l = path length of 1 cm.

Data analysis

The experiment was laid down in a completely randomized design (CRD). The spectrophotometric readings were taken for the blood samples and tabulated. The data on serotonin levels were analyzed using GENSTAT Software (Version 15) and the treatment means compared using LSD. These data were also entered in spread sheets for subsequent generation of graphs and analysis of variance (ANOVA) tables to establish relationships and correlations between serotonin concentrations and W. somnifera root fractions.

Results

Light absorbance by distilled water, ethanol, methanol, n-butanol and xylene extracts

The absorbance values in distilled water ranged from 1.459 to 1.196 (Table 1). However, in Ethanol, the range was 2.301-2.685. In methanol, absorbance varied from 2.017 – 2.405. In addition, the range of absorbance in n-butanol was 1.356- 1.865. Besides, in xylene, absorbance varied from 0.518-0.740. Light absorbance by distilled water, ethanol, methanol, n-butanol and xylene varied significantly (F = 50.2011 P = 1.25x10⁻⁰⁷).

Table 1: Values of absorbance at 295 nm obtained when different sets of Suiz albino mice were treated with root extracts of W. somnifera using different extracting solvents

Distilled water	Ethanol	Methanol	n-butanol	Xylene
1.459	2.685	2.094	1.356	0.740
1.985	2.433	2.405	1.865	0.518
1.930	2.445	1.995	1.789	0.719
1.196	2.301	2.017	1.475	0.629

Concentrations of the metabolites extracted using the selected solvents

The concentration of metabolites extracted from *W. somnifera* using ethanol varied from 0.342×10^{-3} to 0.399×10^{-3} ; methanol (0.297×10^{-3} - 0.358×10^{-3}); n-butanol (0.202×10^{-3} - 0.277×10^{-3}) and xylene (0.077×10^{-3} - 0.110×10^{-3}) (Table 2). The concentrations of the serotonin from mice injected with metabolites from the selected solvents varied significantly ($F = 23.3269$ $P = 0.0000469$).

Table 2: Concentrations of serotonin from mice treated with *W. somnifera* different solvents root extracts multiplied by a factor of 10^{-3} .

Ethanol	Methanol	n-butanol	Xylene
0.399	0.300	0.202	0.110
0.362	0.311	0.277	0.077
0.364	0.358	0.266	0.107
0.342	0.297	0.219	0.093

Comparison between absorbance values of *W. somnifera* root extracts with Panadol

The light absorbance values of extracts with Panadol varied from 0.288 to 0.830. However, in methanol the range was 0.660-2.351. Besides, in ethanol extracts, the absorbance values varied from 0.738-1.986. There was no significant difference in light absorbance values obtained in Panadol, methanol and ethanol extracts ($F = 3.717845$ $P = 0.089$).

Table 3: Comparison between *W. somnifera* root extracts absorbance values and a positive control Panadol on serotonin secretion.

Panadol	Methanol	Ethanol
0.830	2.351	1.986
0.587	0.660	0.738
0.288	1.299	0.777
0.429	1.962	0.972

Conclusion

Extracts from *W. somnifera* have the ability to reduce serotonin levels in plasma. There is need to determine the mechanisms in which the extracts use in reducing serotonin concentration in blood. Mass production of *W. somnifera* extracts need to be carried out.

Discussion

W. somnifera ethanol root extracts had the highest absorbance values followed closely by methanol root extracts, n-butanol and finally xylene root extracts at a wavelength of 295 nm (Table 1). These results differed with a previous study carried out in India (Krutika et al., 2016). The solvents so used could have targeted different metabolites other than those that evoke serotonin secretion and its subsequent recruitment to the platelets (Dey et al., 2014). In addition, Rai et al. (2016) maintained that solvents could have had effect on positive down regulation of

serotonin receptors thus low serotonin absorbance and concentrations as was shown by n-butanol and xylene extracts. The low absorbance values shown by xylene could be explained by the interaction the solvents has with the pigments present in the metabolites (Jayanthi et al., 2014).

The relationship between absorbance and concentration of the metabolites are presented in Fig. 2. The higher the concentration, the higher was the absorbance values. This agreed with study carried out by Ramakanth et al. (2018) on relieving pain in humans using extracts from *W. somnifera*. Similarity in the extracted metabolites may have contributed to the observed results (Nabeel et al., 2013).

Serotonin concentration from ethanol extracts was the highest compared to other solvents (Table 2). This indicated that it was able to recruit serotonin to the site of tissue trauma more effectively than the other root extracts (Giri, 2013). This was also confirmed by the high absorbance values obtained in the current study. However, these results differed with a previous study where methanol had the highest concentration (Alam et al., 2015). This may be explained by differences in the accumulated metabolites in the plants used in the two studies (Ramin et al., 2018).

After heat-treatment of the two best performing extracts, it was observed that methanol extracts produced higher serotonin concentrations than ethanol. This agreed with previous studies carried out elsewhere (Kauray et al., 2013; Mohamed et al., 2014). This could be explained by differences in polarity that solvents obtain when they gain some heat (Kedia and Chattarjia, 2018). In addition, secondary metabolites extracted using n-butanol and xylene showed very low levels of serotonin absorbance and thus concentration compared to methanol and ethanol extracts. This differed with a previous study where the two solvents gave higher concentrations than methanol. This may attributed to the geographical differences in which the plants from which the metabolites were extracted were obtained (Kamal et al., 2017). Dar et al. (2015), asserted that the environment in which *W. somnifera* grow dictates the type and composition of the secondary metabolites it accumulates (Tanna et al., 2014).

Serotonin obtained using ethanol and methanol extracts produced higher absorbance values than those obtained using Panadol (Table 3). This disagreed with a previous study by Umadevi et al. (2016). This may be attributed to variations in the extracted metabolites (Bharathi et al., 2015). Although Panadol was expected to inhibit serotonin secretion more than the former, the values obtained proved otherwise probably due to individual animal internal factors (Wadhwa et al., 2015). In addition, this could also be due to the number of serotonin receptors on individual animal down regulated by Panadol as it does not fully inhibit serotonin secretion (Alam et al., 2015).

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How to cite this article: Githaiga B. M, Lelmen E, Mwangi E. M, Waithaka P. M, Effects of withania somnifera root extracts on serotonin secretion in suiz albino mice, *J Pharm Biolog Sci*. October-December, 2018;6(4):109-113