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The Positive Effect of Astocalm Plus on Stress Excitatory & Inhibitory Neurotransmitters

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A B S T R A C T

The present article describe the effect of a Astocalm plus tablet having anxiolytic effect proved by Monoamine Oxidase inhibition (MAO), Selective Serotonin Re-absorption Inhibition (SSRI), hippocampal region activation, neurite formation, GABAergic activity. The clinical validation has further proved the effect of the formulation in modifying salivary cortisol and salivary amylase in human volunteers during morning and noon hours. The herbs used in the formulation are extremely safe for oral usage. The details of the study findings and statistical significance established by U test and Sign test are presented in the article.

Keywords: Nootropic, herbal stress, anxiety and Siddha, herbal anti-depressants.

INTRODUCTION

Brain development and evolution of bipedalism from quadrupedalismin*Homo sapiens* (human being) is assumed to haveoccurredsimultaneously [1]. The vertical alignment of the spinal cord helps the brain to occupy the top position in the vertebral column and which provides several survival advantages to man in the course of evolution with reference to recognize and deal his environment for the purpose of reward -risk mitigation [2].

Super - evolution of human brain has certainly brought not only the ability to relish life greatly for man but also it brings griefs and anxiety in human life that are either real and or anticipatory/imaginary [3]. The present day life of human being is filled with frets and threats; some are real and while some are imaginary. Human feelings, fear, faith and reasons are largely governed by several hormonal/chemicals constituents in the body collectively referred as neurotransmitters. The neurotransmitters are further broadly divided into two sub categories such as excitatory and inhibitory neurotransmitters [4].

Derailment of the excitatory and inhibitory neurotransmitters often bring several changes in the mind and body of human being [5]. Acetylcholine, epinephrine (adrenaline), glutamate (GABA), histamine, dopamine are examples for excitatory neurotransmitters while serotonin and cortisol are example for inhibitory neurotransmitters in mammalian system. On the other hand, alpha amylase represent sympathetic- adrenal modulatory enzyme do have a specific role in

mind-body harmony. Theneurotransmitters modulate the central nervous system (CNS), autonomic nervous system (ANS), sympathetic nervous system (SNS) and parasympathetic nervous system (PNS).

Aberration in axonal networking due to wrecked neurotransmission will cause emotional changes, anxiety, stress, spatial navigation and cognition [6]. Persistence of the above condition may affect the brain chemistry and anatomy which would progressively results in causing the pathological condition called depression. Further, the same condition also may result in amnesia and progressive dementia.

Amnesia is different from dementia where the neuronal modulation in hippocampal region of the brain may lead to the acquisition of new knowledge or may affect the retrieval of pre-stored information in the brain and such conditions are medically described as anterograde amnesia and retrograde amnesia.

Monoamine oxidase (MAO) catalyze the oxidation of predominantly the excitatory neurotransmitters such as dopamine and epinephrine and also impede the inhibitory hormone serotonin and thereby cause sleep disorder [7].

Anxiety, stress and depression are although may appear as different orbitsinthe same medical spectrum but certainly such conditions would affect the memory and learning ability as well. Stress and anxiety would severely affect the attention threshold as well as the focus and intent to knowledge acquisition. The learning or knowledge when impaired so shall it would affect the process of memory consolidation and memory retrieval.

In allopathic system, treatment for stress, anxiety, depression and problem of memory are largely achieved through a variety of drugs such as sedative agents, monoamine oxidase inhibitors, selective serotonin reabsorption inhibitors (SSRIs) [8, 9], activation of hippocampal region, GABAergic preparations, acetylcholinesterase inhibitors, cortisol modulators, anti-inflammatory agents, pain relievers etc. Most of the drugs known to produce irreversible damage and thereforeare used with great caution [10]. Further the target mechanism of each group of the drug is quite different and therefore multiple drugs targeting different anxiety eliciting factors may be required to put anxiety to rest and such pharmacological approach may cause serious, irreversible side effects.

Herbal preparations are known to improve human brain power and the best examples are *Centellaasiatica*, *Bacopamonnierri* [11, 12]. Further many herbal preparations are reported to have benefits such as anxiolytics, nootropics, sedative, specific effect on elevating excitatory hormones, analgesic, well-being effect etc. however most of the herb/herbal preparations are not studied in detail and the findings are credible enough.

In the present paper, we report the elaborate scientific and clinical studies done on Astocalm plus such as MAO inhibition, SSRA inhibition, and activation of hippocampal cells, neurite formation and GABAergic effect. Further the effect of Astocalm plus in modulating morning and evening salivary cortisol and salivary amylase enzyme in healthy volunteers was also performed in order to predict the possible clinical effect of the preparation. Findings are presented in the article.

Materials and Methods

Details of Astocalm Plus

Astocalm plus is a proprietary Siddha medicine which is composed of 200mg of Withaniasomnifera extract, 150 mg of Bacopamonnieri, and 50 mg each of Nardostachysjatamansi, Myristicafragrans, Syzygiumaromaticum.

Mono Amine Oxidase (MAO) inhibition assay

MAO inhibition was performed by following a standard procedure [13]. In brief MAO enzyme was prepared in Tris-Hcl buffer, pH7.4, at 0.1U/ml. Serotonin, dopamine and norepinephrine prepared in Tris-Hcl buffer was used as substrate. 1mM. Phynylethyamine was used as standard for the present study.

The MAO enzyme 50µl was loaded into 96 well micro titre plate along with 50 µl of the substrates. Blank wells were maintained with buffer alone excluding the substrates. The entire reaction mixture was incubated at 37°C for 30 minutes and then 50 µl of 1M HCl was added to stop the reaction andthen 100 µl of 2m MPEA solution was added to every well including the blank wells and again the plate was incubated at 37C for 30 minutes. The colour formed at the end of the reaction was measured at 492 nm using plate reader. MAO activity was calculated by subtracting the absorbance value of blank vis-à-vis sample wells by following standard procedure.

For studying the effect of Astocalm plus in inhibiting MAO enzyme, the concentrations of Astocalm plus such as 5, 10, 15 and 20 µl was added to the enzyme before the addition of substrate and incubated for 10 minutes and then the substrate was added.

Specific effect on MAO-A and MAO-B

The MAO A and B enzymes at 50 µl were prepared in potassium phosphate buffer and was added to 96 well plate along with the substrates (50 µl) such as Kynuramine for MAO-A and Benzylamine for MAO-B enzyme. The reaction mixture was incubated at 37C for 30 minutes and then 100 µl of NADH (nicotinamide adenine dinucleotide) solution was added to the reaction mixture. And then the reaction mixture was read at 340 nm using microplate reader.

To study the effect of Astocalm plus, Astocalm plus at 5,10,15 and 20 micrograms were treated with the enzyme for 10 minutes prior to the addition of the substrate and then the test was carried out as described above. Direct effect of Astocalm plus on NADH was performed to avoid any possible error.

Effect on SSRAI

Dulbecco's modified eagle medium (DMEM) with high glucose, L-glutamine, 10% FBS and 1% pencillin-streptomycin was prepared and maintained the temperature of the medium to 37C in 96 well cell culture plate. The hippocampal neuron cells at a density of 10,000 cell per well was seeded and incubated at 37C at 5%Co2 for 48 hrs or until the cell reach confluency of 90% [14]. The cells were then washed with HBSS buffer twice and then serotonin at a concentration of 0.01-10µM prepared in HBSS buffer was added to the cells and incubated for 30 minutes at 37C with 5%CO2. After which, the medium was removed, the cells were washed with ice-cold TBS and then the cells were lysed in TBS with a cocktail of protease inhibitor to release the accumulated serotonin from the cells. The amount of serotonin accumulated by Astocalm plus treated and untreated hippocampal cells was assayed by ELISA.

Effect on Hippocampus cells

Hippocampal cells were grown in DMEM with FBS, penicillin-streptomycin solution in the ratio of 89:10:1 and the cells were incubated at 37°C, 5% CO2 until cells reach 70-80% confluence.

The cells were then removed, from the broth, washed with PBS, added trypsin-EDTA solution to detach the cells from the flask and centrifuge the cells at 2000 rpm for 5 minutes.

Measured the cells using a haemocytometer and then plated into 96 well plate and incubated for 24 hrs using the cell culture broth.

After 24 hours, Astocalm extract was added into cell culture plate in triplicate at different concentration such as 10, 20, 30 and 50 mg per mL and incubated further for 48 hrs. After incubation, 20 µl of MTT reagent was added to each well and incubated further for 4 hours.

The medium was then removed and 150 µl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals formed by MTT reduction.

Absorbance at 570 nm was read using a microplate reader and IC50 value was calculated from the OD difference between test and control.

Effect on neurite formation in Hippocampal cells

In brief the Astocalm extract treated cells were washed with PBS and fixed with 4% paraformaldehyde for 20 minutes and then treated with 0.1% Triton X-100 for 10 minutes and then the cells were stained with Hoechst 33342 [15]. Theextent of neurite outgrowth and percentage of neurite formation were qualitatively recorded. Diazepam treated cells were used as control.

Effect on Gamma-aminobutyric acid (GABA) activity in hippocampal cells

The Astocalm plus treated hippocampal cells were incubated with 100 µL of GABA assay buffer, followed by 50 µL of the GABA standard solution or the cell culture broth alone and incubated at room temperature for 30 minutes [16]. After incubation, the plate was read at 450nm using an ELISA plate reader. The standard curve of GABA was used for calculation.

Clinical Evaluation

The effect of Astocalm plus on salivary amylase and salivary cortisol in human healthy volunteer was assayed. The study was approved by institutional ethics committee and subsequently registered with CTRI/2023/10/058179.

Collection of saliva sample

Saliva samples were collected from 20 healthy volunteers who provided written consent to participate in the study by signing ICF. The saliva samples were collected from volunteers at 6 AM and 4 PM on day 1 and day 2 (pre-

treatment stage) and similarly the saliva samples were collected on day 3 and day 4 after the administration of Astocalm plus (post treatment).

Cotton roll was placed in the salivette device and the same was kept over the tongue for 1 minute or till the cotton roll is saturated by saliva. The cotton roll with saliva samples was stored at − 70°C [17]. Prior to assay the sample was allowed to thaw and then centrifuged for 10 min at 2000g and 4°C. The sample was used for salivary amylase assay and cortisol assay.

Salivaryamylase assay

Formation of maltose from starch by amylase enzyme in the saliva was studied by following the method of Bernfeld [18]. The rate of enzyme activity was estimated by the amount of substrate(s) that was utilized by the enzyme and the amount of the end product formed in the given unit time.

Maltose reacts with alkaline dinitrosalicylic acid (DNSA) and would give an orange colour. The maltose thus formed is assayed by stoichiometric method and the same was used to estimate the amount of maltose formed.

The saliva sample thus collected was centrifuged at 3000 rpm for 15 min in 1 ml distilled water and the supernatant was diluted further with distilled water at the ratio of 1:10 (saliva 0.025 ± 0.225 ml distilled water) and the same was used as enzyme source.

Five percentage starch solution IN phosphate buffer (0.1N, pH 6.7) was used as substrate.

To the 5 ml of substrate 0.25 ml of saliva preparation, the source of enzyme was added and incubated for 15 min at 37C and then the reaction was stopped by adding 0.5 ml 2N NaOH 0.5 ml DNSA reagent was then added to the above and mixed well and then the tube was kept in boiling water for 5 min. After cooling, 20 ml distilled water was added and thecolorchangewas measured at 520 nm. A standard graph of maltose was prepared and used as reference to measure the corresponding amount of maltose formed per ml of saliva (mg/ml).

Cortisol assay by ELISA

Cortisol present in the saliva sample was assayed by ELISA [19] following the competition reaction between an unlabelled antigen present in the standard, control, and samples and the enzyme labelled antigen in a microplate which has limited antibody binding sites. The intensity of colour formed was inversely proportional to the concentration of cortisol present in the sample.

RESULT

Mono Amine Oxidase (MAO) assay

Substrate specific MAO activity vis-à-vis inhibition of MAOafter treatment with Astocalm plus.

10 to 15% inhibition of MAO was observed when Astocalm extract was tested and Astocalm plus showed concentration dependent activity, Table-1.

Table-1:

Test details	**% MAO activity**		
	Serotonin	**Dopamine**	**Norepinephrine**
Control	55	62	57
Test	**% MAO inhibition**		
Astocalm plus 5 µg	9	4	7
Astocalm plus 10 µg	11	7	9
Astocalm plus 15 µg	11	9	12
Astocalm plus 20 µg	13	10	15

Specific effect on MAO-A and MAO-B

Astocalm plus exhibited inhibitory effect on MAO- A and MAO-B enzymes comparable with the activity curcumin and EGCG. Although positive control drugs showed greater activity but none of the drugs are in use due to irreversible side effects, Table-2.

Table-2:

Test details	Concentration in µg	% MAO-A inhibition	% MAO-B inhibition
Astocalm extract	5	7	6
	10	11	10
	15	14	16
	20	16	16
Curcumin	20	21	22
EGCG	20	27	35
Clorgyline	2	51	58
Selegiline	2	38	21

Effect on SSRAI

Astocalm plus exhibited SSRAI and the concentration did not correlate with the inhibitory effect, Table-3.

Table-3:

Test details/ µg/ml	SSRAIs activity in %
Astocalm extract 10	31
Astocalm extract 20	34
Astocalm extract 30	35
Astocalm extract 40	36
Astocalm extract 50	41
Fluoxetine 1	70

Neuroprotective of effect of Astocalm extract on Hippocampus cells by MTT

Astocalm plus did not show any significant cytotoxic effect on hippocampal cells clearly indicating neuro protective value in the light its effect in increasing 10% new neural growth during direct examination with Hoechst 33342 staining, Table-4.

Table-4:

Sl. No	Test concentration (mg/ml)	% inhibition
1	10	8
2	20	11
3	30	12
4	50	11
5	Untreated control	-

Effect of Astocalm plus on Gamma-aminobutyric acid (GABA) activity in hippocampal cells

Astocalm plus extract increased GABAergic activity in hippocampal cells and the increased showed a marginal correlation with concentration of Astocalm plus, Table-5.

Table-5:

Test details	Dose (µg/g)	Control (pg/mL)	Experimental (pg/mL)	% Change
Muscimol (positive control)	10	100	160	+60%
DMSO (negative control)	50	100	60	-40%
Astocalm extract	100	100	111	+11%
Astocalm extract	200	100	118	+18%
Astocalm extract	300	100	121	+21%
Astocalm extract	500	100	125	+25%

Clinical evaluation of Astocalm plus on salivary amylase

The salivary amylase profile (unit/mg) in all 20 volunteers before treatment showed consistency between the days (day 1 & 2) confirming a stable catecholaminergic activity. Astocalm plus treatment has significantly reduced the salivary amylase profile both on day 3 & day 4 compared to the respective values of the enzyme on day 1 & day 2, Table-6.

Table-6:

volunteers	Pre- treatment/ Salivary amylase value						Post treatment / Salivary amylase value					
	Day 1		Day 2		Avg. Day 1, 2 6.00 am	Avg. Day 1,2 4.00 am	Day 3		Day 4		Avg. Day 1 ,2 6.00 am	Avg. Day 1,2 4.00 am
	6.00 am	4.00 pm	6.00 am	4.00 pm			6.00 am	4.00 pm	6.00 am	4.00 pm		
1	121	132	130	133	125.5	132.5	99	111	86	89	92.5	100
2	611	655	630	700	620.5	677.5	411	521	345	411	378	466
3	1250	1200	1010	1280	1130	1240	1000	1000	800	800	900	900
4	720	715	734	640	727	677.5	511	456	412	341	461.5	398.5
5	688	700	691	650	689.5	675	489	567	371	440	430	503.5
6	311	400	326	388	318.5	394	200	301	143	281	171.5	291
7	211	388	226	392	218.5	390	167	213	101	185	134	199
8	414	422	444	411	429	416.5	300	365	211	288	255.5	326.5
9	520	611	538	628	529	619.5	321	555	300	410	310.5	482.5
10	736	800	736	891	736	845.5	457	600	311	451	384	525.5
11	1001	950	1001	931	1001	940.5	711	711	546	600	628.5	655.5
12	911	912	911	900	911	906	633	567	444	554	538.5	560.5
13	888	900	888	889	888	894.5	671	688	501	451	586	569.5
14	901	892	901	900	901	896	567	711	500	456	533.5	583.5
15	105	126	105	122	105	124	72	100	64	66	68	83
16	152	170	152	168	152	169	100	143	78	81	89	112
17	501	608	501	611	501	609.5	311	567	211	444	261	505.5
18	674	692	674	673	674	682.5	411	551	299	451	355	501
19	326	400	326	401	326	400.5	201	299	165	167	183	233
20	481	501	481	511	481	506	288	346	189	254	238.5	300

Table-7: Statistical significance of salivary amylase profile in 20 volunteers between day 1 and day 2, before treatment, 6.00AM value – Mann Whitney U- test

Before treatment		S1 values	S1 Ranks	S2 values	S2 Ranks
Sample 1Day 6 am values	Sample 2 Day 2 6 am values				
121	130	105	1.5	105	1.5
611	630	121	3	130	4
1250	1010	152	5.5	152	5.5
720	734	211	7	226	8
688	691	311	9	326	11
311	326	326	11	326	11
211	226	414	13	444	14
414	444	481	15.5	481	15.5
520	538	501	17.5	501	17.5
736	736	520	19	538	20
1001	1001	611	21	630	22
911	911	674	23.5	674	23.5
888	888	688	25	691	26
901	901	720	27	734	28
105	105	736	29.5	736	29.5
152	152	888	31.5	888	31.5
501	501	901	33.5	901	33.5
674	674	911	35.5	911	35.5
326	326	1001	37.5	1001	37.5
481	481	1250	40	1010	39

Significance level- 0.05, two tailed test

Sample 1	Sample 2	Sample 1+2
Sum of ranks – 401	Sum of ranks – 414	Sum of ranks – 820
Mean of ranks- 20.3	Mean of ranks- 20.7	Mean of ranks- 20.5
Expected sum of ranks- 410	Expected sum of ranks- 410	Standard deviation- 36.9685
Expected mean of ranks- 20.5	Expected mean of ranks- 20.5	
U value- 204	U value- 196	
Expected U value- 200	Expected U value- 200	

The U value is 196, the critical value of U at $p<0.05$ is 127, the result is not significant at $p<0.05$

Result 2- Z ratio

The Z score is 0.09468, the p value is 0.92828, result is not significant at $p<0.05$

The result of salivary amylase profile of all 20 volunteers before treatment, on day 1 & day 2 at 6.00am were similar, identical and concordant.

Table-8: Statistical significance of salivary amylase profile in 20 volunteers between day 1 and day 2, before treatment, 4.00AM value - Mann Whitney U- test

Before treatment		S1 values	S1 Ranks	S2 values	S2 Ranks
Sample 1Day 4 pm values	Sample 2 Day 2 4 pm values				
132	133	126	2	122	1
655	700	132	3	133	4
1200	1280	170	6	168	5
715	640	388	7.5	388	7.5
700	650	400	10.5	392	9
400	388	400	10.5	401	12
388	392	422	14	411	13
422	411	501	15	511	16
611	628	608	17	611	18.5
600	891	611	18.5	628	20
950	931	655	23	640	21
912	900	692	25	650	22
900	889	700	26.5	673	24
892	900	715	28	700	26.5
126	122	800	29	889	30
170	168	892	32	891	31
608	611	900	34	900	34
692	673	912	36	900	34
400	401	950	38	931	37
501	511	1200	39	1280	40

Significance level- 0.05, two tailed test

Sample 1	Sample 2	Sample 1+2
Sum of ranks – 414.5	Sum of ranks – 405.5	Sum of ranks – 820
Mean of ranks- 20.72	Mean of ranks- 20.27	Mean of ranks- 20.5
Expected sum of ranks- 410	Expected sum of ranks- 410	Standard deviation- 36.9685
Expected mean of ranks- 20.5	Expected mean of ranks- 20.5	
U value- 195.5	U value- 204.5	
Expected U value- 200	Expected U value- 200	

The U value is 195.5, the critical value of U at $p<0.05$ is 127, the result is not significant at $p<0.05$

Result 2- Z ratio

The Z score is 0.1082, the p value is 0.9124, result is not significant at $p<0.05$

The result of salivary amylase profile of all 20 volunteers before treatment on day 1 & day 2 at 4.00pm were similar, identical and concordant.

Statistical significance of Astocalm plus treatment on morning salivary amylase – Sign test

Positive treatment outcome = 20/20

Z-score Calculation

$z = 4.47214$, p-value is $< .00001$. The result is significant at $p < 0.05$.

Statistical significance of Astocalm plus treatment on evening salivary amylase – Sign test

Positive treatment outcome = 20/20

Z-score Calculation

$Z = 4.47214$, p-value is $.< .00001$, the result is significant at $p < 0.05$.

Salivary cortisol profile in 20 volunteers before and after Astocalm plus treatment (nmol/L)

All the 20 volunteers showed stable and comparable salivary cortisol value at 6.00am and 4.00 pm, before treatment, confirming the stable HPA axis activity. Astocalm plus treatment (both 1st day and 2nd day of treatment) has reduced significantly the salivary cortisol on day 3 & day 4, Table-9.

Table-9:

Volunteers	Pre- treatment/ Salivary Cortisol value						Post treatment / Salivary cortisol value					
	Day 1		Day 2		Avg. Day 1, 2 6.00 am	Avg. Day 1,2 4.00 am	Day 3		Day 4		Avg. Day 1 ,2 6.00 am	Avg. Day 1,2 4.00 am
	6.00 am	4.00 pm	6.00 am	4.00 pm			6.00 am	4.00 pm	6.00 am	4.00 pm		
1	4.7	5.2	4.5	5.2	4.6	5.2	3.1	3.1	3	2.7	3.05	2.9
2	3.9	5.4	4.1	5.3	4	5.35	3.2	3.3	3	2.3	3.1	2.8
3	5.1	5.9	5.2	6.1	5.15	6	4.4	3.2	4	3.2	4.2	3.2
4	4.9	5.1	4.8	5	4.85	5.05	3.9	3.3	3.4	3	3.65	3.15
5	5.4	4.3	5.5	4.1	5.45	4.2	3.7	2.8	3.6	2	3.65	2.4
6	5.3	4.6	5.4	4.3	5.35	4.45	3.2	3.2	3.3	3	3.25	3.1
7	3.8	5.1	3.7	5	3.75	5.05	2.5	3.1	2.3	2	2.4	2.55
8	3.6	5.2	3.7	5	3.65	5.1	3.2	2.8	3	2.4	3.1	2.6
9	4.2	3.9	4.3	4	4.25	3.95	2.7	3.1	2.4	3	2.55	3.05
10	4.3	5.6	4.4	5.7	4.35	5.65	3.1	3	3	2.6	3.05	2.8
11	5.1	5.8	5.2	5.9	5.15	5.85	4.6	4.1	4.5	3.6	4.55	3.85
12	5.2	5.2	5.3	5.1	5.25	5.15	3.2	4	3.2	3	3.2	3.5
13	4.8	5.1	4.9	4.9	4.85	5	3.2	3.1	3.3	3.2	3.25	3.15
14	5.6	5.6	5.7	5.4	5.65	5.5	2.6	3.2	2.2	3.3	2.4	3.25
15	2.5	2.1	3.1	2.2	2.8	2.15	2.5	1.7	2.4	1.4	2.45	1.55
16	2.5	2.6	2.5	2.5	2.5	2.55	2.1	1.8	2	2	2.05	1.9
17	2.9	3.1	3	3.2	2.95	3.15	1.8	3.1	1.5	3	1.65	3.05
18	3.1	3.3	3.2	3.1	3.15	3.2	2.5	2.6	3	3	2.75	2.8
19	2.6	3.5	2.5	3.1	2.55	3.3	2.3	2.1	2.5	2	2.4	2.05
20	2.4	3.7	2.3	3.1	2.35	3.4	2.1	2.1	2	2	2.05	2.05

Table-10: Statistical significance of salivary cortisol values in 20 volunteers on day 1 and day 2, before treatment, 6.00 am value – Mann Whitney U-test

Before treatment		S1 values	S1 Ranks	S2 values	S2 Ranks
Sample 1Day 6 am values	Sample 2 Day 2 6 am values				
4.7	4.5	2.4	2	2.3	1
3.9	4.1	2.5	4.5	2.5	4.5
5.1	5.2	2.5	4.5	2.5	4.5
4.9	4.8	2.6	7	3	9
5.4	5.5	2.9	8	3.1	10.5
5.3	5.4	3.1	10.5	3.2	12
3.8	3.7	3.6	13	3.7	14.5
3.6	3.7	3.8	16	3.7	14.5
4.2	4.3	3.9	17	4.1	18
4.3	4.4	4.2	19	4.3	20.5
5.1	5.2	4.3	20.5	4.4	22
5.2	5.3	4.7	24	4.5	23
4.8	4.9	4.8	25.5	4.8	25.5
5.6	5.7	4.9	27.5	4.9	27.5
2.5	3.1	5.1	29.5	5.2	32
2.5	2.5	5.1	29.5	5.2	32
2.9	3	5.2	32	5.3	34.5
3.1	3.2	5.3	34.5	5.4	36.5
2.6	2.5	5.4	36.5	5.5	38
2.4	2.3	5.6	39	5.7	40

Significance level- 0.05, two tailed test

Sample 1	Sample 2	Sample 1+2
Sum of ranks – 400 Mean of ranks- 20 Expected sum of ranks- 410 Expected mean of ranks- 20.5 U value- 210 Expected U value- 200	Sum of ranks – 420 Mean of ranks- 21 Expected sum of ranks- 410 Expected mean of ranks- 20.5 U value- 190 Expected U value- 200	Sum of ranks – 820 Mean of ranks- 20.5 Standard deviation- 36.9685

The U-value is 190. The critical value of U at $p < .05$ is 127. Therefore, the result is not significant at $p < .05$.
The Z-Score is -0.25698. The p-value is .79486. The result is not significant at $p < .05$.
The result of salivary cortisol profile in all 20 volunteers before treatment on day 1 & day 2 at 6.00am were similar, identical and concordant.

Table-11: Statistical significance of salivary cortisol values in 20 volunteers on day 1 and day 2, before treatment, 4.00AM value – Mann Whitney U-test

Before treatment		S1 values	S1 Ranks	S2 values	S2 Ranks
Sample 1Day 4 pm values	Sample 2 Day 2 4 pm values				
5.2	5.2	2.1	1	2.2	2
5.4	5.3	2.6	4	2.5	3
5.9	6.1	3.1	6.5	3.1	6.5
5.1	5	3.3	10	3.1	6.5
4.3	4.1	3.5	11	3.1	6.5
4.6	4.3	3.7	12	3.2	9
5.1	5	3.9	13	4	14
5.2	5	4.3	16.5	4.1	15
3.9	4	4.6	18	4.3	16.5
5.6	5.7	5.1	24.5	4.9	19
5.8	5.9	5.1	24.5	5	21
5.2	5.1	5.1	24.5	5	21
5.1	4.9	5.2	28.5	5	21
5.6	5.4	5.2	28.5	5.1	24.5
2.1	2.2	5.2	28.5	5.2	28.5
2.6	2.5	5.4	32.5	5.3	31
3.1	3.2	5.6	34.5	5.4	32.5
3.3	3.1	5.6	34.5	5.7	36
3.5	3.1	5.8	37	5.9	38.5
3.7	3.1	5.9	38.5	6.1	40

Significance level- 0.05, two tailed test

Sample 1	Sample 2	Sample 1+2
Sum of ranks: 428 Mean of ranks: 21.4 Expected sum of ranks: 410 Expected mean of ranks: 20.5 U-value: 182 Expected U-value: 200	Sum of ranks: 392 Mean of ranks: 19.6 Expected sum of ranks: 410 Expected mean of ranks: 20.5 U-value: 218 Expected U-value: 200	Sum of ranks: 820 Mean of ranks: 20.5 Standard Deviation: 36.9685

The U-value is 182. The critical value of U at $p < .05$ is 127. Therefore, the result is not significant at $p < .05$.
Result 2- Z ratio
The Z-Score is 0.47338. The p-value is .63836. The result is not significant at $p < .05$.
The result of salivary cortisol profile of all 20 volunteers before treatment on day 1 & day 2 at 4.00pm were similar, identical and concordant.

Statistical significance of Astocalm plus treatment on morning salivary cortisol – Sign test
Positive treatment outcome = 20/20
Z-score Calculation
$Z = 4.47214$, p-value is < .00001, the result is significant at $p < 0.05$

Statistical significance of Astocalm plus treatment on evening salivary cortisol – Sign test
Positive treatment outcome = 20/20

Z-score Calculation
Z = 4.47214, p-value is < .00001, the result is significant at $p < 0.05$.

DISCUSSION

With the advancement of modern medical science, the mental health issues have started to gain pivotal importance in medicine because the mental health issues besides affecting the health and quality of life also preludes the elicitation and aggravation of various chronic medical conditions such as psoriasis, diabetes mellitus, arthritis, cardio vascular diseases etc.

Further the anxiety and stress are the most common symptoms of mental health problem (depression, amnesia, dementia, alzheimer's disease). And anxiety and stress also have their strong origin in immune related complications that are either chronic (auto-immune) or transient in nature. Perpetual existence of stress and anxiety may also cause serious cognitive disability, reasoning, and memory impairment.

Forthe management of stress, anxiety, depression, memory fading and problems with cognition, requires drugs where the safety is as much important as efficacy because most of the allopathic preparations used for the above conditions are known to cause several irreversible side effects and worsening of the condition, when such preparations are used for prolonged period of time. Long duration treatment is necessary with such drugs to deal the above mental conditions.

The pathological events occurring in the brain starts with the cleavage/ depletion of several neurotransmitters such as dopamine, oxytocin, serotonin, melatonin, epinephrine, nor epinephrine, amylase, cortisol etc. Further GABAergic activity also would decrease and that would result progressive dis-functioning of hippocampus neurite network.

Herbal preparations have been used by man since time immemorial for mental wellbeing and some such preparations are Cocaine alkaloid prepared from the plant Erythroxylum coca and cannabinoids from Cannabis sativa, popularly called as Marijuana. The concept of psychedelic benefits and its addiction have attracted mankind since ages [20].

Mono Amine Oxidase (MAO) are group of enzymes that catalyse the oxidation of monoamines (neurotransmitters) such as dopamine, norepinephrine, serotonin etc., in the brain. Inhibition of MAO can achieve increased levels of neurotransmitters in the brain space which would improve the cognition and alertness of mind and body; suppressed by their depletion. MAO is one of the primary causesfor the neuropsychiatric disorders such as depression, anxiety etc., and therefore the inhibition of the enzyme is necessary to preserve the level of several neurotransmitters in the brain sufficient to keep the mental health in robust state.

There are two main types of MAOs such as MAO-A and MAO-B [21]. MAO-A is responsible for deamination of serotonin, melatonin, epinephrine, norepinephrine, and dopamine. The MAO-B is responsible for the deamination of tyramine and also dopamine. The allopathic drugs that are popularly used for the inhibition of MAO are Selegilline, Safinamide, Tranylcypromine, Isocarboxazide etc., among the two isomers of MAO, MAO- A resides in the intestine while MAO- B is present in the platelets and in the brain where dopaminergic neurons are present. Early adverse reactions of MAO inhibitors are insomnia, sedation, ortho-static hypertension, dizziness, nausea. The median adverse events due to the usage of MAO inhibitors are weight gain, odema, muscle pain, myoclonus, Paresthesia. Serious medical conditions due to MAO inhibitor is hypersensitive crisis and serotonin syndrome [22].

Astocalm plus is a poly-herbal preparation, formulated with the inclusion of herbal actives with scientifically reportedeffect/ empirical usage in the Indian system of medicine and which was tested for MAO inhibition. The study was conducted at two levels

1. The activity inhibition of MAO by Astocalm plus when the enzyme was fed with dopamine, serotonin and norepinephrine.
2. The effect of Astocalm plus on MAO- A and MAO - B by using NADH as cofactor

Our present study reveal that Astocalm plus exhibited uniform inhibitory effect on MAO when the neurotransmitters used as substrate suggesting the possible therapeutic value during clinical usage. Subsequent experiment using NADH as cofactor also reveal the interfering effect of MAO deamination biochemistry indicating the possible MAO specific activity of Astocalm plus.

Hippocampal cells progressively get deteriorated due to aging where the selective reabsorption of serotonin and the neurite damage also would cause anxiety, depression and associated medical conditions up to the spectrum of AZD.

Therefore protection ofthe hippocampal cells and possible inducement to the hippocampal region to stimulate neurite formation is needed to increase neural communication network. Initially the Astocalm plus was tested for it's a non-cytotoxic effect on hippocampal cells by MTT assay.

Astocalm plus did not show any cytotoxic effect and interestingly increased the neurite growth when studied by Hoechst 33342 staining. Neuritesare the extension of neuronal process such as axons and dendrites which form the essential part of the communication network among neurons.

After establishing the effect of Astocalm plus on MAO and neurite formation in hippocampal cells we went further to establish the possible effect on GABA (gamma-aminobutyric acid).

GABA is an inhibitory neurotransmitter in the central nervous system. GABAergic interneurons play a crucial role in regulating the excitability of the hippocampal circuitry. Therefore we proposed to study the expression of GABA and the receptors in response to Astocalm plus intervention on GABAergic signalling.

Anxiolytic drugs in general would increase the GABAergic transmission in the brain, which would results in increased GABA expression. Due to the complex relationship between GABA expression in hippocampal cells at cell culture level. Different drugs may have anxiolytic effect and also may have same target site but different mechanism of action, therefore the activity established for Astocalm plus at cell culture level can only provide directional therapeutic value.

For example, the benzodiazepines such as diazepam and lorazepam, the common anxiolytic drugs [23] would enhance GABAergic transmission by directly binding to GABA-A receptors, leading to an increased GABA expression. On the other hand the Buspirone and Pregabalin modulate GABAergic transmission but through different mechanisms.

For instance, buspirone is a partial agonist of serotonin 5-HT1A receptors [24], which can indirectly increase GABAergic transmission, while pregabalin binds to the α2-δ subunit of voltage-gated calcium channels, leading to a reduction in the release of neurotransmitters including GABA. Astocalm plus significantly increased GABA from 11 to 25% from a concentration ranging from 100 to 500 microgram per gram.

Although the exact underlying mechanism of action of Astocalm plus could not be established but its possible role in GABAergic activity in hippocampal region could be established by the study.

Serotonin is the chemical messenger that sends messages between nerve cells and throughout the body and thereby control several crucial role in various brain functions, including mood regulation, sleep, appetite, and social behaviour.

Imbalances in serotonin levels can lead to various mental health disorders such as depression, anxiety, and obsessive-compulsive disorder (OCD) [25]. Serotonin reabsorption is a normal physiological process that occurs in cells and is essential for the proper functioning of the nervous system but heavy re-absorption can cause depletion of serotonin available in the synaptic cleft for neurotransmission. Therefore, limiting re-absorption can offer a solution to depression, anxiety and other associated conditions.

The serotonin depletion occur in our body mainly due to body isn't producing the required level serotonin and or body could not utilize the serotonin. SSRIs like Venlafaxine, Nardil etc., would block the enzyme that oxidize serotonin while some other group of drugs would block the serotonin receptor and thereby impair reabsorption.

Astocalm plus appears to have 'dual door blockage' mechanism where at one level it prevent the MAO enzyme that oxidizes monoamines and also the study of Astocalm plus on hippocampal cells suggest that serotonin receptor also the Astocalm plus may be blocking and thereby ensuring the availability of serotonin.

The clinical evaluation of Astocalm plus was carried out in human subjects in order to understand its effect on salivary cortisol and salivary amylase. Two day follow up study on healthy volunteers before and after treatment with Astocalm plus was done by collecting salivary samples at 6.00 am and 4.00 pm on both days.

The Mann- whitney U test and Z value showed the amylase and cortisol values of the respective volunteers between the days (day 1 & day 2) before treatment did not vary much and such variation was statistically insignificant.

The treatment of Astocalm plus on the above set of volunteers was administered orally on day 3 and day 4. The statistical significance of the data before and after treatment both for cortisol and amylase at 6.00 am and 4.00 pm was

arrived. Both the salivary cortisol profile and salivary amylase profile in all the volunteers before and after treatment showed statistical significance clearly suggesting the salivary amylase and cortisol modulating effect of Astocalm plus.

Based on our literature search, we found that among herbal drug category, Astocalm plus appears to be first herbal drug that has undergone elaborate screening for its anxiolytic effect starting from MAO inhibition, hippocampal cell safety, neurite stimulation, GABAergic effect, SSRI, besides salivary cortisol and salivary amylase modulation. Considering the safety and rapid metabolism and poor accumulation Astocalm plus may be considered for the management of stress, anxiety and depression.

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