Bio-Therapeutical study of Herbal dietary supplement composed of Mangosteen, Siberian Ginseng, Maca root, Elderberry, Raspberry, Sourcherry, Black currant, Ganoderma and Moringa.

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RESEARCH DESIGN

1. Processing
2. Formulation,
3. Nutritional analysis
4. Phytochemical Analysis
5. Quantitative Estimation of phytochemical
6. Primary in-vitro studies

6.1 Antimicrobial assay,
6.2 Anti-oxidant assay,
6.3 Anti inflammatory assay

7. Qualitative analysis by FT-IR and HPLC
8. Stability Studies
9. In-vivo studies

9.1 Oral Toxicity test
9.2 Acute Inflammatory test (In Progress)
9.3 Anti-diabetic test (In Progress)
9.4 Anti-cancer Test (In Progress)

1. PROCESSING AND EXTRACT PREPARATION

1. The plant parts that were taken for study were Whole fruit (Mangosteen), Roots (Siberian Ginseng, Berries (sour cherry, Elderberry, Raspberry, Black currant), whole leaves (Moringa) and Whole macro-fungus (Ganoderma).
2. Samples were shade dried to reduce moisture content. The samples were then milled and sieved to achieve uniform size of the powdered sample.
3. The portion of moringa leaf powder taken for formulation was 36% and rest plant parts portions were taken in equivalent ratio (8% each).
4. The powdered sample and 100% water taken in ratio of 1gm : 20ml and the mixture were kept in amber colored bottle and kept in shaking condition at room temperature for 48 hours. 0.1% of Sodium Benzoate was also added to inhibit microbial growth.
5. After 48 hours, the sample was filtered with Whatman No.1 filter paper. The filtrate was again dessicated by Rotatory evaporator and the concentrated extract was collected in collection flask.
6. The extract was reconstituted to 0.1 mg/ml by DMSO solvent for further experimental analysis.

2. FORMULATION INGREDIENTS (Aqueous Extract)

Mangosteen
(*Garcinia mangostana*)

Siberian ginseng
(*Eleutherococcus senticosus*)

Maca root
(*Lepidium meyenii*)

Ganoderma
(*Ganoderma lucidum*)

Elderberry
(*Sambucus*)

Raspberry
(*Rubus idaeus*)

Sour Cherry
(*Prunus cerasus*)

Black currant
(*Ribes nigrum*)

Moringa
(*Moringa oleifera*)

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3. NUTRITIONAL CHARACTERISTICS OF HERBAL FORMULATION

TEST RESULTS				
Test Description	Method	Unit of Measurement	Daily Values	**Results**
Energy	By calculation	Kcal/100g	-	**357.49**
Total Carbohydrate	AOAC 986.25	g/100 g	19	**72.71**
Total Protein	IS 7219:1973	g/100 g	15	**10.79**
Total Fat	AOAC 922.06	g/100g	48	**2.61**
Total Sugar	DGHS Manual 04	g/100g	-	**13.51**
Total Dietary Fiber	AOAC 985.29	g/100g	9	**20.97**
Insoluble Dietary Fiber	AOAC 991.43	g/100g	-	**19.39**
Soluble Dietary Fiber	AOAC 991.43	g/100g	-	**1.58**
MUFA (%TOTAL)	AOAC 996.01	mg/100g	-	**1.17**
PUFA (%TOTAL)	AOAC 996.01	mg/100g	-	**0.79**
Zinc	AOAC 2011.14	g/100g	11	**4.26**
Iron	AOAC 2011.14	mg/100g	29	**140.18**
Calcium (Ca)	AOAC 2011.14	mg/100g	-	**1709.2**
Potassium (K)	**AOAC 2011.14**	**mg/100g**	-	**1359.7**

TEST RESULTS				
Test Description	Method	Unit of Measurement	Daily Values	**Results**
Magnesium (Mg)	AOAC 2011.14	mg/100g	-	**334.50**
Sodium (Na)	AOAC 2011.14	mg/100 g	-	**173.0**
Copper (Cu)	AOAC 2011.14	mg/1000 g	-	**16.90**
Vitamin A	AOAC 2001.13	IU/100g	25	**<100**
Vitamin C	AOAC 996.06	mg/100g	10	**<10**
Vitamin B12	AOAC 2011.09	µg/100g	2	**2.31**
Vitamin E	AOAC 2011.13	mg/100g	10	**7.06**
Vitamin K	AOAC 999.15	µg/100g	<100	**165.39**
Vitamin B7	As per protocol	µg/100g	20	**<10**
Vitamin D2	AOAC 2016.05	µg/100g	-	**<0.10**
Vitamin D3	AOAC 2016.05	µg/100g	-	**<1.0**
Vitamin B1	BS EN 14122-2003	mg/100g	-	**<0.1**
Vitamin B6	EN 14164:2008	mg/100g	-	**<0.1**
Folic acid	**As per protocol**	**µg/100g**	**4**	**<10**

9.1 IN-VIVO STUDIES (ORAL TOXICITY TEST)

1. Male Wistar albino rats weighing 150 – 200 gm were used for the study. The animals and ethical clearance for the study were obtained from National Institute of Nutrition (NIN), Hyderabad, India.
2. The experimental protocols were reviewed by Institutional Animal Ethics Committee (IAEC) and all the procedures were in accordance with IAEC.
3. On arrival, the animals were placed at random and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at a temperature of 24±2 C and relative humidity (RH) of 65%±5%. A 12:12 light: day cycle was followed.
4. All the animals fasted for 4 hours with free access to water.
5. Control animals were given 1ml of 0.5% Carboxy Methyl Cellulose (CMC). Test animals were administered orally at 10mg/mL initially and observed for 3 days.
6. If Mortality had occurred in more than 50% sample size, then the dose considered as toxic dose. If mortality had occurred in less than 40% sample size, the experiment was repeated again to confirm toxic effect.

Serial Number	Sample	Inference	Dose
1.	Herbal Dietary mix (DMSO)	No significant change in general behavior and no mortality was observed in the experimental animal	5000mg/kg

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4. Phytochemical Analysis (Qualitative) (Fransworth 2008)

Test for	Test Name	Positive Observation	Test Result
Alkaloid	Wagner's Test	Reddish brown coloration/precipitate	+
Carbohydrate	Molisch Test	Formation of red or dull violet colour at the interface of the two layers	+++
Glycosides	Keller Killiani test	Brown ring at the interface, Brown-greenish ring may form.	+++
Flavonoids	Alkaline reagent test	Intense yellow coloration that becomes colorless on addition of dil.HCl.	+++
Phenol	Aq. FeCl3 Test	Deep blue/Black coloration	+
Amino acid	Ninhydrin Test	Formation of purple color.	++
Saponin	Foam Test	Formation of persistence foam	+
Tannin	Braymer's Test	Formation of blue or greenish colour	+
Terpenoids	Salkowaski Test	Formation of reddish brown precipitate	+
Quinone	Conc. HCl Test	Formation of yellow precipitate or colouration.	++
Steroids	Liebermann-Burchard test	Formation of greenish blue colour	++
Coumarin	Alkaline test	Formation of yellow color	++
Resin	Turbidity test	Formation of yellow color	-

(+) POSITIVE Observation **(-) NEGATIVE Observation**

1. Fransworth N., *The Role of Ethanopharmacology in Drug Development. Bioactive Compounds from Plant*, John Wiley & Sons, 2008.

5. Quantitative Estimation of Phytochemicals

Serial number	Phytochemical	Methodology	Standard Equivalent	Quantity
1	Flavonoid (470 nm)	Aluminium chloride spectrophotometric assay. (Pekal 2014).	Quercetin	18.97±0.31 mg Quercetin/g
2	Alkaloid (510 nm)	Bromocresol green spectroscopic method (Shamsa et al. 2007)	Atropine	3.19±0.26 mg Atropine/g
3	Phenolic (550 nm)	Folin - Ciocalteu method Ainsworth et al. 2007)	Gallic acid	45.37±0.32 mg Gallic acid/g
4	Tannin (725 nm)	Folin - Ciocalteu method (Okuda et al. 1982)	Tannic acid	1.67±0.19 mg Tannic acid/g

1. Ainsworth EA, Gillespie KM. Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. Nat Protoc. 2007;2(4):875-7. doi: 10.1038/nprot.2007.102. PMID: 17446889.
2. Okuda, T.; Mori, K.; Shiota, M. Effects of the interaction of tannins with coexisting substances. III. Formation and solubilization of precipitates with alkaloids (Jap.) Yakugaku Zasshi 102:854 (1982).
3. Pękal, A., Pyrzynska, K. Evaluation of Aluminium Complexation Reaction for Flavonoid Content Assay. *Food Anal. Methods* **7,** 1776–1782 (2014). https://doi.org/10.1007/s12161-014-9814-x
4. Shamsa Fadhil , Monsef Hamid Reza , Ghamooshi Rouhollah and Verdian Rizi Mohammad Reza , 2007. Spectrophotometric Determination of Total Alkaloids in Peganum harmala L. Using Bromocresol Green. Research Journal of Phytochemistry, 1: 79-82.

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6.1 Invitro Studies (Anti-microbial studies) Anti-Bacterial and Anti-Fungal (Boyan et al. 2008)

Serial Number	Pathogenic Bacteria	Zone of inhibition (in mm)
1	*Escherichia coli*	8.5±0.20
2	*Pseudomonas aeruginosa*	11.25±0.35
3	*Staphylococcus aureus*	6.56±0.33
4	*Salmonella typhi*	2.3±0.16
5	*Streptococcus pyogenes*	0.00±0.00
6	*Mycobacterium sp.*	9.66±0.14
7	*Bacillus cereus*	18.26±0.76
8	*Enterococcus faecalis*	0.00±0.00
9	*Clostridium botulinum*	0.00±0.00
10	*Yersinia sp.*	1.97±0.45

Serial Number	Pathogenic Fungus	Zone of inhibition (in mm)
1	*Aspergillus niger*	7.35±0.40
2	*Aspergillus flavus*	6.8±0.65
3	*Candida albicans*	2.45±0.15
4	*Mucor sp.*	9.29±0.33
5	*Microsporium*	0.00±0.00

1. Boyan Bonev, James Hooper, Judicaël Parisot, Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method, Journal of Antimicrobial Chemotherapy, Volume 61, Issue 6, June 2008, Pages 1295–1301, https://doi.org/10.1093/jac/dkn090

6.2.1 Anti-Oxidant assay (DPPH reduction assay) (Brand-Williams et al. 1995)

Serial Number	Sample	Concentration	(%age of inhibition)
1.	Ascorbic acid (Standard)	0.1mg/mL	79.58±1.47%
2.	Herbal Dietary mix (DMSO)	0.1mg/mL	67.89±1.88%

1. Brand-Williams,W.; Cuvelier, M.E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. Food Sci. Technol. **1995**, 28, 25–30.

6.2.2 Anti-Oxidant assay (Phosphomolybdate assay) (Khatoon et al. 2013)

Serial Number	Sample	Concentration	(%age of inhibition)
1.	Ascorbic acid (Standard)	0.1mg/mL	48.76±0.51%
2.	Herbal Dietary mix (DMSO)	0.1mg/mL	42.49±0.43%

1. Khatoon, M., Islam, E., Islam, R. *et al.* Estimation of total phenol and *in vitro* antioxidant activity of *Albizia procera* leaves. *BMC Res Notes* **6,** 121 (2013). https://doi.org/10.1186/1756-0500-6-121

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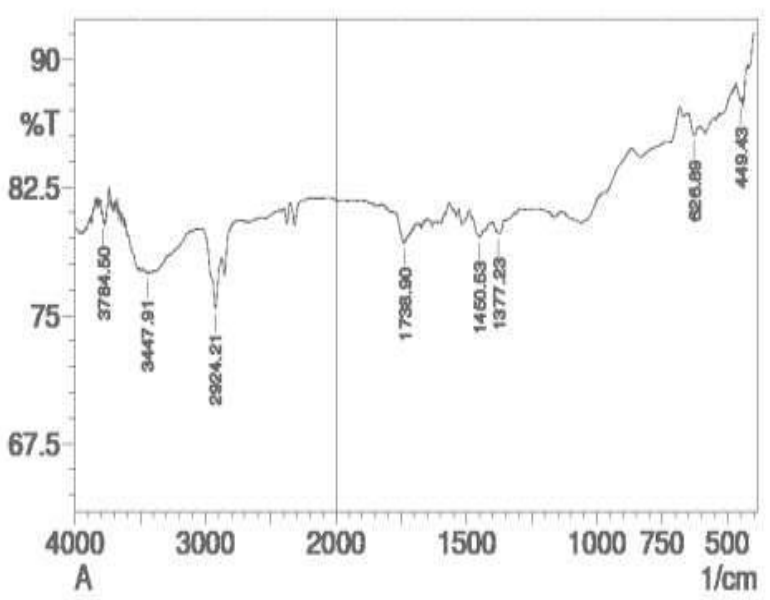
6.3 Invitro Studies (Anti-inflammatory activity) (Banerjee et al. 2014)

Serial Number	Sample	Concentration	(%age of inhibition)
1.	Indomethacin (Standard)	0.1mg/mL	72.09% ±1.76%
2.	Herbal Dietary mix (DMSO)	0.1mg/mL	62.78% ±1.33%

1. Banerjee S, Chanda A, Adhikari A, Das A, Biswas S. Evaluation of Phytochemical Screening and Anti Inflammatory Activity of Leaves and Stem of Mikania scandens (L.) Wild. *Ann Med Health Sci Res*. 2014;4(4):532-536. doi:10.4103/2141-9248.139302

7.1 Qualitative determination of Functional group FT-IR analysis (Primpke et al. 2018)



FTIR spectral peak values and functional groups Identification

Wave number (cm-1)	Functional group
3784.50	-O-H group
3447.91	-N-H group
2924.21	C-H stretching
1738.90	C=O carbonyl group
1450.93	C-H bending
1377.23	C-H bending
626.89	C-O stretching
449.43	C-O stretching

1. Primpke, S., Wirth, M., Lorenz, C. *et al.* Reference database design for the automated analysis of microplastic samples based on Fourier transform infrared (FTIR) spectroscopy. *Anal Bioanal Chem* **410,** 5131–5141 (2018). https://doi.org/10.1007/s00216-018-1156-x

7.2 HPLC analysis (Qualitative Compound Profilling)

The qualitative compound profiling was carried out by Normal High Performance Chromatographic (Normal HPLC) procedure to find out different compounds resolute into constituent fraction having different retention time and peak area. A total of 24 different compounds were obtained

Conditions for HPLC were
Mobile phase: Isocratic type
Mobile Phase : Methanol:Water:Formic Acid = 70 : 29.30 : 0.70
Column Type : C18
Flow rate: 1.5ml/min
Injection volume: 5µL
Run Time : 20 mins
Column Temp: 25°C
Detector λmax: 230nm.

HPLC description:
Build : Waters 2695 Alliance Separation Module.,
Detector: Dual Lambda 2487 UV Detector.,
Column description: Inertsil ODS C-18, 250 x 4.6, 5 micron meter

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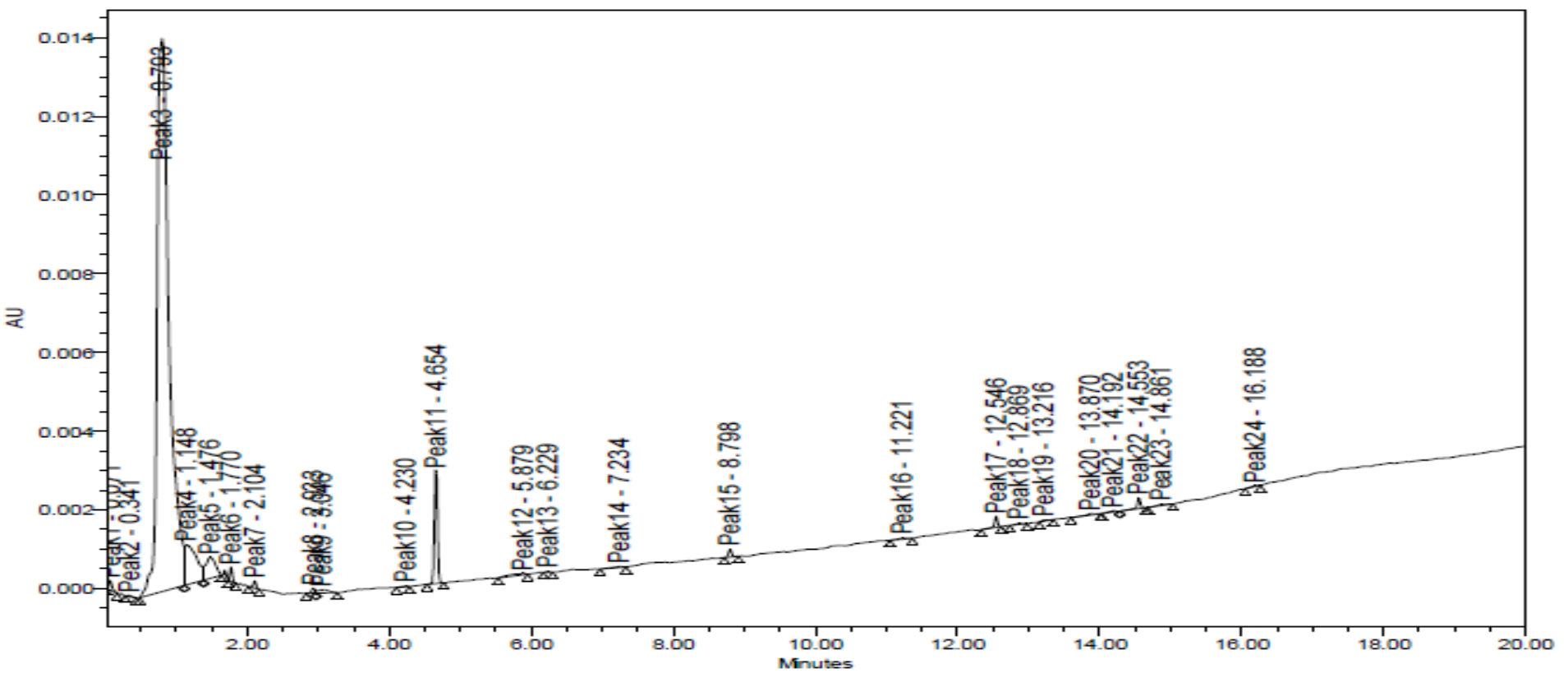
7.2 HPLC analysis (Qualitative Compound Profilling) Continues



HPLC Chromatogram (Peak Development)

Peak name	Retention time	Area	Height	%age of area	USP Resolution	USP tailing	USP plate count
Peak1	0.071	795	229	0.03		1.66	5.52
Peak2	0.341	158	34	0.01	2.07	1.25	93.72
Peak3	0.793	166884	14074	5.65	2.25		147.48
Peak4	1.148	11474	1006	0.39	0.39		8.72
Peak5	1.476	5144	539	0.17	0.35		322.32
Peak6	1.770	915	332	0.03	1.41	1.16	6410.10
Peak7	2.104	534	170	0.02	3.55	1.26	7194.75
Peak8	2.933	427	123	0.01	5.59		3520.59
Peak9	3.046	970	83	0.03	0.29		417.15
Peak10	4.230	151	28	0.01	3.01	0.72	7863.50
Peak11	4.654	8814	2741	0.30	2.92	1.02	34942.34
Peak12	5.879	773	45	0.03	2.39	0.57	645.85
Peak13	6.229	74	22	0.00	0.68	1.05	55538.51
Peak14	7.234	331	33	0.01	5.20	0.68	10588.64
Peak15	8.878	635	188	0.02	8.10	0.86	113113.85
Peak16	11.221	266	34	0.01	13.07	0.88	28419.85
Peak17	12.546	894	262	0.03	7.26	0.61	258667.03
Peak18	12.869	190	32	0.01	2.20	0.65	69510.79
Peak19	13.216	174	33	0.01	1.94	1.56	107290.15
Peak20	13.870	316	38	0.01	3.62	0.55	76328.58
Peak21	14.192	250	35	0.01	1.57	0.68	74059.47
Peak22	14.553	962	254	0.03	2.31	0.60	314245.12
Peak23	14.861	282	34	0.01	2.00	0.96	85040.46
Peak24	16.188	171	25	0.01	6.59	0.57	105822.14

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8. Stability studies

Stability studies refers to the shelf life study of Herbal dietary supplement formulation described above. This assures accurate date for products, ensuring quality, safety and efficacy.

Conditions for testing
Type : Accelarated
Time: 6 months , 12months, 18 months, 24 months
Attributes: Temperature (38 °C ± 2 °C); Relative Humidity (90% ± 5%)
Test Result for study based on **24 MONTHS.**

Serial number	Parametres	Units	Methodology	Limit	Result	Status
1.	Appearance	-	IS 6273(II): 1971	-	Acceptable	PASS
2.	Odour	-	IS 6273 (II): 1971	-	Acceptable	PASS
3.	Texture	-	IS 6273 (II): 1971	-	Acceptable	PASS
4	pH	-	FSSAI 5(2.3):2016	5-7	5.7	PASS
5	Acidity	gm/100gm	FSSAI 5(2.4):2016	2.2-2.9	2.58	PASS
6.	Moisture	gm/100gm	FSSAI 3(8.1):2016	4.5-6.0	5.34	PASS
7.	Total Viable	CFU/gm	IS 5402:2012	<10	2.21	PASS
8.	Yeast & Mold	CFU/gm	IS 5403:1999	<10	0.91	PASS
9.	*E.coli*	Org/gm	IS 5887(I): 1976	Absent	Absent	PASS
10	*Salmonella*	Org/25gm	IS 6579 (I): 2017	Absent	Absent	PASS
11	*Staphylococcus*	Org/gm	IS 5887 (II0: 1976	Absent	Absent	PASS
12	*Coliform*	CFU/gm	IS 5401 (I) :2002	Absent	Absent	PASS

CONCLUSION

The Herbal supplement derived from the aqueous extract of Whole fruit (Mangosteen), Roots (Siberian Ginseng, Berries (sour cherry, Elderberry, Raspberry, Black currant), whole leaves (Moringa) and Whole macro-fungus (Ganoderma) have a good potential anti-microbial potential against pathogenic bacteria and fungus, anti-inflammatory activity and anti-oxidant activity. Higher anti-oxidant activity may be due to the extracts obtained from moringa and mangosteen that have potential to reduce the super oxides and help in better performance of body metabolism thereby reducing oxidative damages and serious ailments such as cancer and liver related diseases. Nutritional assessment also show promising as a better alternative to commercial available food supplements and multi-vitamins. Invivo studies on oral toxicity experiment show that the herbal dietary preparation show no sign of mortality/morbidity even at higher dose concentration upto 5000mg/kg dedicating its safety aspect.

Herbal nutraceutical is used as a powerful instrument in maintaining health and to act against nutritionally induced acute and chronic diseases, thereby promoting optimal health, longevity, and quality of life. Nutritional therapy is a healing system using dietary therapeutics or nutraceuticals as a complementary therapy. This therapy is based on the belief that foods can not only be sources of nutrients and energy but could also provide medicinal benefits.

THANK YOU