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PMNL Cell Phagocytosis Activity Alters with Poly-Herbal Formulation in Diabetic Patients

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Abstract: One of the major complications of diabetes weakens the immune system, which puts the diabetic person at risk for difficult-to-treat and persistent infections and delayed healing of wounds, especially the feet. Phagocytosis by polymorphonuclear leucocytes (PMNL) is the first line of defense against any infection. Diabetics are more prone to long lasting infections due to several reasons, the most important of them is impaired phagocytosis. **We evaluated the change in** Phagocytosis activity by using of a poly-herbal formulation in diabetic patients, Subjects were divided into four groups, Group I (NH), were normal healthy subjects, Group II (DI), were diabetics, group III (TTD) was tolbutamide treated diabetic patients, group IV (HFTD), were diabetic patients receiving combination herbal formulation in the, dosage of 5 g. /Day for 4 weeks. The result was exciting with prescribed formulation since it was found to be effective in the correction of all parameters related to phagocytosis , which are found to be disturbed in diabetic patients leading to an array of persistent infection . The present study could be proved an excellent immunopotiator will be a boon for diabetic patients in the near future for the management of diabetic complication.

Keywords: NMV, PMNL Opsonization, Phagocytosis, Nitro Blue Tetrazolium

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I. INTRODUCTION

Glucose levels are persistently elevated, chemotaxis and phagocytosis are compromised. Chemotaxis is the process by which white cells are attracted to the site of infection, while phagocytosis is the ingestion of bacteria by white cells. Both processes are important in controlling wound infections [3]. Diabetic infections take a longer time to heal because of the delayed macrophage introduction and diminished leukocyte migration, which causes a prolonged inflammatory phase in the wound healing cascade [4]. Phagocytosis by polymorpho nuclear lymphocytes (PMNL) is the first line of defense against any infection [5]. Diabetics are more prone to long lasting infections due to several reasons, most important of them being impaired phagocytosis [6]. Reasons for impaired phagocytosis can be envisioned as –Impairment in the activity of Na^+/K^+ - ATPase in diabetic PMNL, the enzyme responsible for maintaining the membrane potential of these cells .Operated calcium homeostasis due to Increase in the cytosolic calcium concentration. Operated calcium messenger system due to decrease in the activities of Ca^{2+}/Mg^{2+} - ATPase, camp Phosphodiesterase and phospholipase in these cells. Aberrations in contact angle, particles internalized, and % PMNL, participation in phagocytosis [7]. The operated Phagocytic Index as indicated by Nitro Blue Tetrazolium Reduction Test [8]. The evaluating phagocytosis activity of the formulation by assaying all the above parameters before and after administration of the herbal formulation was totally a novel approach not reported hitherto.

II. MATERIALS & METHODS

Drugs and Chemicals

All the chemicals, drugs and reagents used in this study were standard and analytical grade.

Study design

Subjects were divided into four groups of 40 subjects in each group. The group I was normal healthy subjects, Group II were diabetics with a fasting blood glucose level of 160-175 mg/dl. , group III were tolbutamide (oral hypoglycemic drug) treated diabetic patients, group IV were diabetic patients receiving combination herbal formulation in the, dosage of 5 g. /Day for 4 weeks. The formulation was administered one hour before food to prevent any interference with its absorption..Diabetic patients were contacted and convinced from two government hospitals-Hamidia Hospital, Bhopal and K.N. Katju hospital, Bhopal. The patients were examined at the time of their visit to the outpatient Department (OPD) and consisted of all men (Mean age, 45 $\pm$ 12.8 years). No patients had any severe infection nor were on any sort of medication. The volunteers were maintained as per norms of center ethics committee on human research (CECHR) and local ethics committees.

Plant materials

Specimens of *Gymnema sylvestre* , *Asparagus racemosus* , *Withania somnifera* , *Andrographis paniculata, Tinospora cordifolia* and *Terminalia arjuna* were procured from Sanjivani, Department of Forest, Government of Madhya Pradesh, Link Road Bhopal(M.P.).

Preparation of polar polyherbal formulation

The all plant parts were shed dried and blended and mixed properly until a homogeneous mixture was obtained. The mixture was encapsulated to ensure blinding, uniformity of administration and for the convenience of patients (Table 1).

Table – 1 The combination of herbs was as follows:

S.No.	Botanical Name of the plant	Common (Indian) Name	Part used	Quantity in the formulation
1.	***Gymnema Sylvestre (Asclepiadaceae)***	Gudmar	Leaves	100 g.
2.	***Asparagus racemosus (Liliaceae)***	Shatavari	Root	100 g.
3.	***Withania somnifera (Solanaceae)***	Ashwagandha	Root	50 g.
4.	***Andrographis paniculata (Acanthaceae)***	Kalmegh	Root	100 g.
5.	***Tinospora cordifolia (Menispermaceae)***	Guduchi	Root	100 g
6.	***Terminalia arjuna (Combretaceae)***	Arjun	Bark	50 g

Isolation of PMNL (polymorphonuclear leukocyte)

PMNL were isolated by the method of **Boyum (1968) [**9] with slight modifications. 10 to 15 ml fasting blood samples was drawn into heparinized tubes already containing 5% dextran (prepared in 0.9% NaCl; use 2 ml 5% dextran for every 10ml of blood).. This preparation contained 97-99% PMNL when viewed under phase contrast microscope. (TarLzeif, AXIOVERT-200, NEERI, Nagpur).

Opsonization of bacteria

Opsonized bacteria were obtained by the method previously described by **Nathan *et.al,* (1991) [10],** with certain modifications. Overnight cultures *of S. aureus and E. coli* were formalinized (1% v/v), heated at 65^0 C for 30 min and then centrifuged at 8000 x g for 15 min. The pellet obtained was washed three times with 0.15 M saline. Two volumes of such a suspension of bacteria were then mixed with one volume of respective antibody and incubated at 37^0 C for 30 minutes. Sensitized bacteria thus obtained were centrifuged at 8000 x g for 15 min, washed twice in Hank's balanced salt solution (HBSS) and then re-suspended in HBSS and adjusted to 2 x 10^8 cells/ml.

Evaluation of phagocytosis activity

Assay of Ouabain sensitive and insensitive adenosine triphosphatase

PMNL were suspended in 0.34M sucrose and homogenized with chilled teflon pestle for 1 min which yielded greater than 95% rupture. The suspension was then spun at 600 g for 10 min and the resulting pellet was resuspended in 0.34 M sucrose and used as enzyme source. Membrane ATPase (ouabain sensitive and insensitive) was determined by the method of **the Post and Sen. (1967)** [11**].** The ouabain sensitive ATPase activity was obtained from the difference in total ATPase with ouabain insensitive ATPase activity. The specific activity was expressed as n mol of Pi released/min/mg protein.

Cellular water content

Cellular water content was determined by the method of **Parker (1971)** [12]. Cellular water content was expressed as kg H_2O/kg dry solids.

Estimation of cytosolic calcium concentration

Cytosolic calcium concentration was assayed by the method of **Murphy, *et.al,* (1980)[13]** The absorbance was measured at the wavelength pair, 675 and 685 nm before and after addition of digitonin. The cytosolic free calcium concentration was assumed to be equal to the free ionic calcium concentration of the medium when no net change of calcium occurred upon addition of digitonin.

Measurement of total cell and plasma membrane calcium content:

Calcium was measured using atomic absorption spectrophotometer (Shandon Southern A3400) by the method of **Parker *et.al, (*1967)** [14]. Readings were taken using an atomic absorption spectrophotometer (Shandon southern A3400) using appropriate standards of $CaCO_3$, results were expressed as µg's of calcium/mg protein.

Ca^{2+}/Mg^{2+} - ATPase of the plasma membrane

Plasma membrane fraction was prepared as described in methodology (Chari and nath 1984) and was finely suspended in 0.34 M sucrose. The suspension served as the enzyme source. Ca^{2+}/Mg^{2+} - ATPase activity was assayed by the method of **Lynch and Cheung (1979)** [15] Inorganic phosphate released was measured by the method of **Fiske and Subbarow (1925)** [16] and the blue color developed after 10 min was read at 660 nm. Specific activity was expressed as n mol of Pi liberated/min/mg protein.

Ca^{2+}/Mg^{2+} - ATPase of non-mitochondria-vesicular portion

NMV fraction was prepared as described (Chari and Nath 1984) [19] and served as the enzyme source. NMV Ca^{2+}/Mg^{2+} - ATPase activity was assayed by the method of **David Mclennan (1970)[17].**

Cyclic-3', 5'-adenosinemonophosphate phosphodiesterase activity

cAMP phosphodiesterase activity was assayed by the previously described method of **Butcher *et.al.* (1962)[18].**

Phospholipase-c activity

Phospholipase-c activity using phosphatidylinositol or phosphatidy 1- choline or phosphatidylethanolamine as substrate was assayed by the method described previously by **Ottolenghi (1969 [19].** After development of the color for 30min at 37^0C, the optical density was read at 740 NM Specific activities was expressed as normal of P_i released/min/mg protein.

Measurement of contact angle

Contact angles were measured by the method described previously by **Van Oss *et.al.* (1975) [20].** The measurement was done by turning the rim of the goniometer until one of the hair with the cross-hair was tangential to the drop at the place where it was in contact with the surface. The contact angle was then read on the rim of the goniometer. In all cases the angles of 10 or more sessile drops of physiological saline were measured and the average taken.

Nitroblue tertrazolium reduction

Nitroblue tetrazolium reduction index (phagocytic index) was measured by the method of **Baehner and Nathan** (1968) [21] with slight modifications. The extracts were combined, and the optical density of the purple color of reduced nitroblue tetrazolium (NBT) was determined at 515 nm against a pyridine blank. The optical density of an extract of cells and NBT incubated for 10 sec was also determined, and this value was subtracted from the others as a reagent blank. Resting and phagocytosing values were obtained and the difference (OD per 15 min per 2.5 x 10^6 PMNL) calculated was expressed as phagocytic index.

Statistical analysis

All the grouped data were statistically evaluated and the significance of various treatments was calculated using Student's *t*-test with the help of appropriate statistical software (*SPSS, V8.2*). All the results were expressed as mean S, $P<0.001$ was considered significant [22].

III. RESULTS

Phagocytosis which is the first line of defiance gets disturbed in diabetic patients resulting in an array of infections in them. Phagocytosis is carried out by specific group of cells in the body polymorphonuclear leucocyte (PMNL). The activity of oubain insensitive ATPase associated during phagocytosis in normal PMNL, (control), (91.63 %). PMNL obtained from untreated diabetic patients had quite depressed activity of these enzymes both in resting and phagocytosing state as compared to normal, (control), (Table-2) tolbutamide treated diabetic group did not show any significant recovery

Table: -2 change in the activities ouabain sensitive) and insensitive ATPase of Polymorphonuclear leucocyte (PMNL).

S.no.	Subject **(n=40)**	Na+/K+ - ATPase (ouabain sensitive). mu moles of Pi liberated/min/mg protein		Na+/K+ - ATPase (ouabain insensitive). mu moles of Pi liberated/min/mg protein	
		RESTING	PHAGOCYTING	RESTING	PHAGOCYTING
1	NH	4.57±0.41	0.91±0.09	20.93±2.31	40.11±2.31
2	DI	1.39*±0,17	0.69**±0.19	9.64±1.07*	16.83*±2.70
3	TTD	2.01***±0.76	0.81**±0.11	10.23ns±1. 91	18.19ns±3. 62
4	HFTD	3.95±0,81	0.84±0.2	18.51±1.99	36.11±2.61

*P<0.001 as compared to group-I
**P<0.01 as compared to group-I
***P<0.02 as compared to group-I
Ns= non-significant as compared to group-I

Table: - 3 change in cellular water contents of PMNL during phagocytosis.

s.no	**Subject** n=10	**Water content (Kg water/Kg dry solids)**	
		Resting	Phagocytosis
1	NH	3.08±0,61	4.42±0.61
2	DI	5.57*±0.51	5.97*±1.02
3	TTD	5.06**± 0.90	5.79**±0.57
4	HFTD	3.12**±0.92	4.38**±0.52

*P<0.001 as compared to group-I
**P<0.02 as compared to group-I

The percentage increase being (43.5%) increment in cellular water contents in phagocytosing diabetic PMNL was only (7.2%) PMNL from tolbutamide treated patients' elicited negligible recovery, and the increment in their cellular water content during phagocytosis was (14.48%), encouraging and significant results were observed in the combination drug treated patients. The increase in cellular water content during phagocytosis was nearly the same as observed in normal (PMNL), (control), (40.38%Vs 43.5%). It is evident from the results in Table-3. As shown (fig.-1), the leucocyte (Ca^{2+}) of diabetics recorded significant increment (104±0.2 nmol) as compared to normal (control), (67±0.5nmol),($p<0.001$). The sight, but not an insignificant decrease in total as well as plasma membrane (PM), Calcium was noticed in untreated as well as tolbutamide treated diabetic patients. The values of total as well as PM calcium were observed (fig-2). Table-4 clearly indicates the status of Ca^{2+}/Mg^{2+} ATPase of PMNL under resting condition in diabetics PMNL. There are significant decrement in the activities of plasma membrane (9.16+2.02 nmol), and NMV Ca^{2+}/Mg^{2+} ATPase (2.76+0.16 n mol) as compared to normal (control). In PMNL of herbal drug controlled diabetics, although the PM and NMV $Ca2^{+}/Mg^{2+}$ ATPase recorded significant normalization (20.01+0.43 n mol) and (4.15+0.93 n mol) represent $p<0.001$, results were non- significant in case of tolbutamide treated PMNL. Table-5 reveals the cAMP Phosphodiesterase profile of normal diabetic and treated PMNL in resting state. CAMP Phosphodiesterase activity was significantly low in resting diabetic PMNL (1.22±0.09nmol) as compared to normal (control). Fig-3 represented the fold increase in activity of cAMP Phosphodiesterase of normal diabetic, tolbutamide treated and herbal drug treated PMNL during phagocytosis. The activity of this enzyme rases 2.08 fold in normal phagocytosing PMNL as compared to resting values. The raise was only 1.63 fold in diabetic PMNL. There was a partial recovery in the fold increase of phagocytosis PMNL of controlled diabetic patients, Response with tolbutamide being quite insignificant (1.6 fold) and herbal formulation being appreciable 2.0fold. Table-6, Clearly indicated that the activity of the C-kinase–RE-phaspholipase-C using all the these substrates viz.– phasphatidylinositol(PI),(1.41±0.31nmol), phasphotidylcholine (PC) ,(2.41±0.43nmol), and phasphotidyl ethanolmine (PE) ,(2.13±0.11nmol) was reduced in resting diabetic PMNL as compared to normal using PI, PC, and PE (3.72±0.18nmol, 5.19±0.16nmol,4.73±0.35nmol) respectively

Table-4 Effect of Phagocytosis on the alteration in the activities of Ca2+/Mg2+ ATPase of NMV of PMNL

S.No.	Subjects	Ca2+/Mg2+ -ATPase	
	n=10	Plasma membrane	Non mitochondrial vesicular portion
		(nmol Pi librated/min/mg protein)	
1	NH	21.21+1.20	4.75+0.43
2	DI	9.16+2.02*	2.76+0.16*
3	TTD	11.21+3.09ns	2.79+0.09ns
4	HFTD	20.11+0.43*	4.15+0.93*

*P<0.001 as compared to group-I
ns= non significant as compared to group-I

Fig: - 1, Alteration in cytosolic calcium concentration [Ca2+] of Polymorphonuclear leucocyte(PMNL)

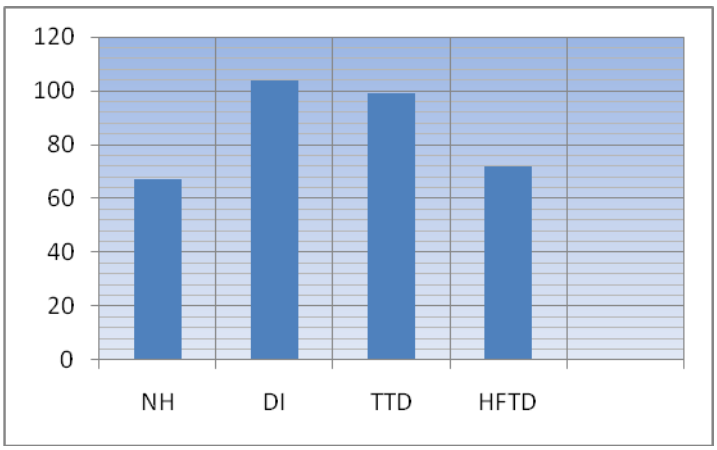


Fig:- 2 , Total Cell and Plasma membrane Calcium content of Polymorphonuclear leucocyte(PMNL) .

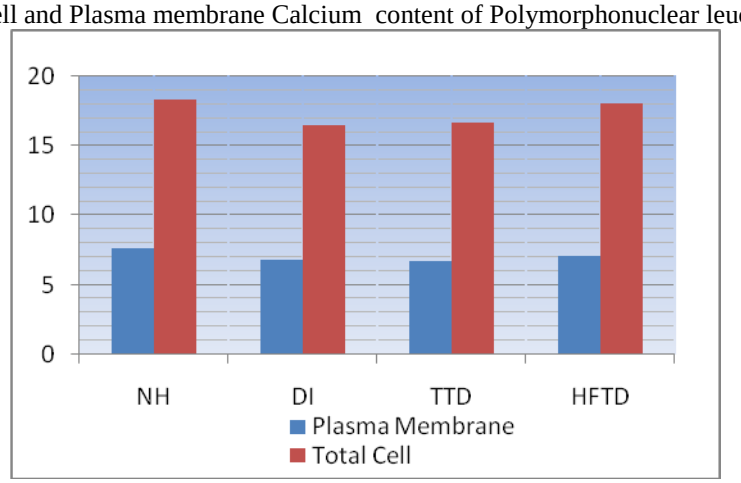


Table:-5 cAMP Phosphodiesterase activity of PMNL in resting state.

s.no.	Subject n=40	cAMP Phosphodiesterase activity (nmol PI liberated/min/mg protein)
1	NH	3.51±0.41
2	DI	1.22*±0.09
3	TTD	2.21**±0.43
4	HFTD	3.19ns±0.56

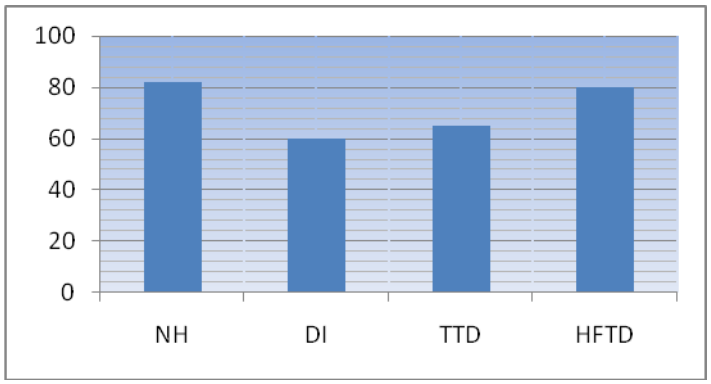
*P<0.001 as compared to group-I
**P<0.02 as compared to group-I
ns= non significant as compared to group-I

Table: - 6 Phospholipase-c activity of PMNL using varied substrate under resting condition

s.no	Subject n=40	Phospholipase-c (n mol of Pi liberated/ min/mg protein) **substrates**		
		Phosphatidylinositol	phosphatidylcholin	Phosphatidylethanolamine
1	NH	3.72±0.18	5.19±0.61	4.73±0.35
2	DI	1.41*±0.31	2.41*±0.43	2.13*±0.11
3	TTD	1.99*±0.31	3.21*±0.72	2.98*±0.71
4	HFTD	3.02*±0.29	4.96*±0.2	4.39**±1.99

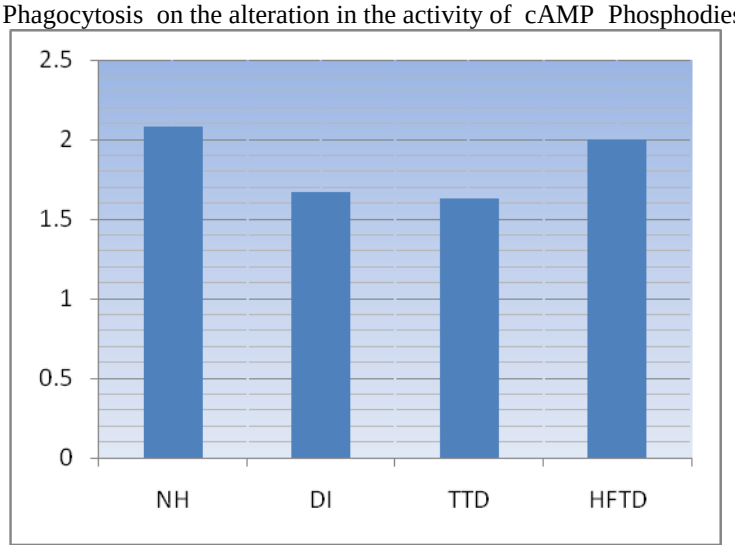
*P<0.001 as compared to group-I
*P<0.02 as compared to group-I

Fig:-3 Effect of Phagocytosis on the alteration in the activities of Ca2+ /Mg2+ ATPase of plasma membrane of PMNL



A partial recovery in phaspholipase –C activity was recoreded in PMNL obtained from tolbutamide treated diabetics. It could also be perceived that the combination herbal drug demonstrated for better response, Using PI, PC, and PE (3.02±0.29nmol, 4.96±0.2nmol, 4.39±1.99nmol) respectively. Fig-4 represented the fold increase in phaspholipase activity during phagocytosis in normal and pathological groups using PI,PC, and PE as substrates fold increase was arrived at by dividing the activity reached during phagocytosis by the respective activities in resting sate . Contact angle of Opsonized *E.coli*. (29˚± 1˚) and *S.aureus* (24.3˚±1˚) were higher as compared to their unopsonised counter parts (26˚±1* and 19.7˚±1˚ respectively). Contact angle was significantly increased in diabetic PMNL (25.2˚±0.1˚) compared to normal (17.20˚±0.1˚) (Fig-5). Phagocytic capability of normal PMNL was higher using Opsonized *E.coli* (4.6±0.36 particles internalized) *S. aureus* (22+0.24 particles internalized), as compared to the Phagocytic capability with unopsonized particles (Table-7).

Fig:- 4 Effect of Phagocytosis on the alteration in the activity of cAMP Phosphodiesterase of PMNL



Among these E.coli demonstrated a higher Phagocytic capability compared to *S. aureus*. the Phagocytic index(NBT reduction assay) estimates the ability of neutrophills and macrophages to produce oxygen radicals (O2,OH-,H2O2). The ability of PMNL and macrophages to kill pathogenic microbes is probably one of

the most important mechanisms of defense NBT reduction assay (Table -8). It is evident that the Phagocytic index of diabetic PMNL, (0.12±0.05) in highly depressed as compared to normal (0.23±0.02).

Fig-5:- comparative hydrophobicity of PMNL as denoted by their contact angles, numbers of subjects are shown in parenthesis.(Contact angle of opsonised and non-opsonised *E.coli*, *S. aureus* were 29˚ +1˚, 26˚+1˚, 19.7˚+1˚)

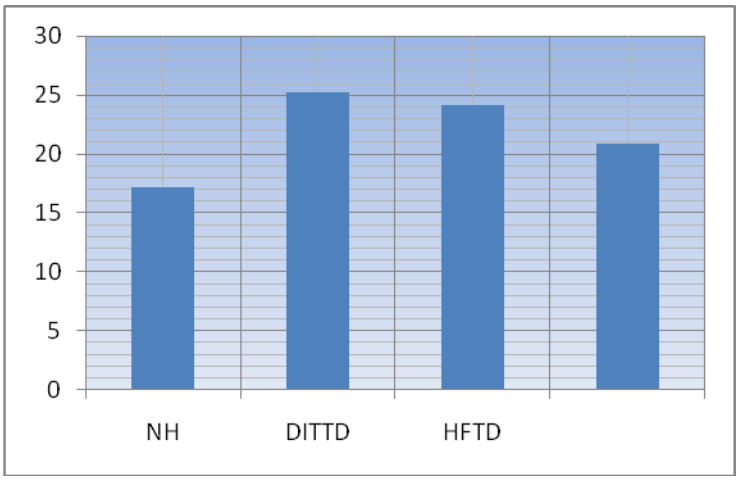


Table -7:comparative Phagocytic capabilities of PMNL with opsonized and unopsonized E.coli and S. aureus.

s.no	subject	**Particles internalized**				**%PMNL participation in Phagocytosis**			
	n=40	***E.coli***		***S. aureus.***		***E.coli***		***S. aureus.***	
		opsonized	unopsonized	opsonized	unopsonized	opsonized	unopsonized	opsonized	unopsonized
		Value represented as mean +SE				**Only mean values represented**			
1	NH	4.6±0.36	2.7±0.27	2.2±0.24	1.3±0.12	89	85	51	43
2	DI	1.7±0.14*	0.32±0.09*	0.21±0.03	0.08±0.01	64	55	11	11
3	TTD	1.8±0.17*	0.34±0.21*	0.23±0.09*	0.07±0.01	65	57	12	12
4	HFTD	4.3±0.16**	1.9±0.12	1.46±0.06*	1.0±0.02	79	74	43	39

*P<0.001 as compared to group-I
**P<0.02 as compared to group-I
***P<0.1 as compared to group-I

TABLE:-8 Changes in Phagocytic index (NBT reduction test of PMNL)

S.no	Subjects n=40	**Phagocytic index** OD=515nm(Phagocytic cells)
1	NH	0.23±0.02
2	DI	0.12±0.05
3	TTD	0.21±0,01
4	HFTD	0.19±0.01

IV. Discussion

Numerous experimental studies based on clinical observation showed that Phagocytic functions of various cells were decreased in diabetics .investigations carried out to understand the pathogensis of these changes in the cholesterol/phaspholipid ratio of the cell membrane due to diabetic infection cause hypercholestrolmia[23] . This has been associated with a decreases in membrane fluidity thus altering several function including the cation transport mechanisms specially Na+/K+ ATPase activity [24]. Decreases in the activity of Na+/K+ ATPase have been observed in diabetic macrophages and PMNL's. *Androphecous paniculata roots* which were included in the herbal formulation have claimed to be an activator of Na+/K+ ATPase activity [25,26]. A recovery in the activity of Na+/K+ ATPase in diabetic PMNL consequent to the administration of 5grams/day of the herbal formulation for four weeks also demonstrate significantly recovery in the cellular water contents. Simultaneously activity Ca2+/Mg2+ ATPase was also assayed. It is the enzymes

responsible for the mobalizaation of calcium from intercellular stores. It is also become imperative to determine the total as well as plasma membrane calcium content. It was successful attempts where the formulation containg *Aspargous reacemosous* and *Tinospora cardifolia* could implement a correction in the cell [Ca2+]c. which had been found to be disturb in diabetic mellitus . it is well recognized that in diabetic leucocyte mobility chemotoxins and phagocytosis are grossly decreased [27].There are no doubt that plats like *Aspargous racemosous* and *Tinospora cordifolia* have been proved to be immune potentiating as well as antioxidant in the vast literature assayable on medicinal plants. Evidence of opsonisation affecting a raise in bacterial contact angle and associated phagocytic ability with normal PMNL is in according to the finding of Vann Oss 1970. Finding of difference in contact angle (delta c) between normal PMNL and examine PMNL. E.coli. being greater than *S.aureus* eliciting higher bacterial inter-normalization in proportion to delta-c(contact angle) was in conformation with the concept that hydrophobicity determines Phagocytic capability [28] Ethanolic extract of *T. cardifolia* has been shown to improve phagocytic function in mice [29] .Root of *A. racemosous* and *T. cordifolia* are well known for their immune-potentiating effect .but till date no work was ever done on studying the effect of herbal drugs on enzymes, which are so important in maintaining a proper biochemical balance inside the cell. Present study where roots of above plants were blended and included in the formulation were quite effective since they could demonstrate a significant recovery by increasing activity of the enzymes Phaspholipase-C, in HFTD, PMNL during resting followed by an enchased increase during phagocytosis. The combination herbal formulation demonstrate a significantly recovery in all the above mentioned parameters

V. Conclusion

The immune system is the major defense against infection is compromised in diabetes. Present study was aimed deeply into the various parameters responsible for phagocytosis by, PMNL. result were exciting with the combination herbal formulation since it was found to be effective in the correction of all parameters related to phagocytosis ,which are found to be disturbed in diabetic patients. On the basis of all results of study, it could be concluded that the formulation of six plants exerted a significant immune-potentiating effect. These effects are due to different types of active principles, each with single or diverse range of biological activities working in synergism, which serve as good adjuvant in the present armamentarium of anti-diabetic drugs.

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