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Review Article

Invitro Assessment of Herbal Extracts in Periodontics Using Antibacterial, Anti-inflammatory, Immunomodulatory and Antioxidant Activities: An Insight

Liya Anil and KL Vandana*

Department of Periodontics, College of Dental Sciences, Davangere, India

***Corresponding Author:** KL Vandana, Department of Periodontics, College of Dental Sciences, Davangere, India.

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Abstract

Many bacteria, in particular have unpredictable susceptibilities to antimicrobial agents and their susceptibilities can be measured *in vitro* to help guide the selection of the most appropriate antimicrobial agent. The various *in vitro* analysis used for this purpose are antibacterial, anti-inflammatory, immuomodulatory and antioxidant activities. The objective of this paper is to provide a brief review on various invitro activities such as antibacterial, anti-inflammatory, immuomodulatory and antioxidant effect on few herbal extracts used in periodontics and also to evaluate the invitro activities of polyherbal gel (HiOra-SG) and oleozone gel.

Keywords: Jasminum; Syzgium; Neem Oil; Olive Oil; Anti-Inflammatory; Antibacterial; Immunomodulatory; *Invitro*; Periodontitis; Herbs

Introduction

Herbal medicine has been widely studied and investigated during the past years both in the field of medicine and dentistry. Lack of drug resistance which scores over the allopathic medicine with respect to antibacterial action is the main advantage. The multipotent therapeutic benefits provided by herbal extracts are most beneficial to treat chronic diseases like periodontitis where in there is inflammation, infection, oxidative and immunomodulatory insults. Few herbal extracts which are commonly used in periodontics are clove, jasmine, licorice, neem, spreading hogweed, triphala, vanatulsi, aloe vera and olive oil.

In periodontics, there are few clinical studies where in herbal extracts have been used either along with or without gold standard chlorhexidine. Various forms have been tried as mouthwash, irrigation and gelforms as local drug delivery. However, these studies lack invitro assessment of the herbal extracts in terms of anti-inflammatory, antioxidant and immunomodulatory assays. The most commonly available form of antibacterial evaluation of herbal extract is Minimum Inhibitory Concentration (MIC), whereas two other components of antibacterial assay such as Minimum Bacterial Concentration (MBC) and Time Kill Curve (TKC) are rarely evaluated.

The invitro approaches to assess the herbal extract are not available as a readymade comprehensive information. Medline search using keywords periodontitis, herbs, antibacterial, immunomodulatory, antioxidant, antiinflammatory and invitro revealed minimal literature. Hence, in this paper we attempted to present the antibacterial, anti-inflammatory, antioxidant and immunomodulatory

tests conducted to evaluate and estimate the multipotent therapeutic benefits provided by herbal extracts.

The objective of this paper is to provide a brief review on various invitro activities such as antibacterial, anti-inflammatory, immuomodulatory and antioxidant effect on few herbal extracts used in periodontics and also to evaluate the invitro activities of polyherbal gel (HiOra-SG) and oleozone gel.

Need for antibacterial test

Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC)

Many bacteria, in particular have unpredictable susceptibilities in antimicrobial agents, and their susceptibilities can be measured invitro to help guide the selection of the most appropriate antimicrobial agent.

The antibiotic sensitivity of microorganism is needed to select the appropriate antibiotic agent. Depending on concentration, the antibiotic can be bacteriostatic or bactericidal. The inhibitory potential of antibiotic is evaluated by filter paper disc [1], agar and broth dilution method [2] and the dilution method. For determining Minimum Inhibitory Concentration (MIC) and Minimum Bacterial Concentration (MBC), the dilution method is used. MIC is the antimicrobial agent in its least concentration which prevents the microbial growth. MBC is the least antimicrobial agent to kill the microorganisms [3,4]. They are determined by using agar medium free of antibiotic into which the sample to be tested is inoculated which shows no turbidity or growth. To avoid an additional time and use of petri dishes in agar medium, Dilution Tube Method (DTM) is introduced wherein only broth medium is used in tubes instead of agar medium for MIC and MBC determination which is cheaper and easier.

Antibiotic sensitivity testing or antibiotic susceptibility testing

Antibiotic susceptibility testing or antibiotic sensitivity testing is generally done as the bacteria may exhibit resistance to few antibiotics. It is the assessment of susceptibility of bacteria to a given antibiotic. This allows the clinician to select the choice of antibiotics effective against an infection as directed for therapeutic benefit than empirical therapy.

In the medical laboratory, sensitivity testing is done either by culture methods in which bacteria are exposed to antibiotics or genetic methods to evaluate if bacteria have genes which are responsible for resistance. In culture methods, the diameter of zone of inhibition which represents areas without bacteria growth are measured around discs coated with antibiotics on agar culture dishes which would be evenly inoculated with bacteria.

E-test and PCR (Polymerase Chain Reaction) methods

Since the discovery of β-lactum antibiotic pencillin, antibiotic susceptibility testing is done. Earlier phenotypic methods are either culture or inhibition. The ongoing research is directed to improve current methods to make them more accurate and faster and new method of testing such as microfluidics are being developed.

The empiric therapy refers to prescription of an antibiotics on the basis of medical guidelines to treat the disease symptoms. This is simply based on the basic knowledge about causative bacteria for an infection to which an antibiotic will be prescribed so that causative bacteria may be sensitive or resistant in a geographical area [5]. However the bacteria may have intrinsic resistance to same antibiotics and resistance may be transmitted from plasmids. Antibiotic sensitivity testing is advisable to treat the infection successfully.

The importance of antibacterial testing

The antimicrobial resistance (AMR) is regarded as one of the health threats by WHO. In approximately 2.6 million clinical infections, anti-microbial resistance bacteria are implicated and 35,000 human deaths yearly due to antibacterial resistance. For this reason, microbial susceptibility testing by MIC is on a regular basis to track microbial resistance in different microbial populations. In MIC testing, a series of antimicrobial concentrations on a two-fold scale are bypassed to bacterial isolate. The MIC is said to be lowest concentration that inhibits the visible growth of the infecting agent completely as examined by the unaided eye after 18-24 hours of incubation period using a standard inoculum of 105 CFY/ml approximately. In this protocol, the pharmacokinetics parameter is generally the serum concentration of the anti-infective agent and MIC is almost exclusively the pharmacodynamics parameter [6]. MBC is the lowest concentration of the antibiotic which causes complete destruction of the infective agent, has been considered for the same purpose [7].

To determine the success of antimicrobial therapy, the complex interactions between the host, infecting agent and administered antimicrobial agent are considered. In a given clinical scenario, the interaction between them are complex that reflects as the high variability in the dose response relationship. This dose response variability can be minimized by considering the dose and drug selection on the invitro parameters such as MIC and the pharmacokinetic parameter to access the drug serum concentration. The determination of these key characteristics will prevent either therapeutic failure or emergence of resistant strains.

The clinical implication of MIC has never been understood by most of us. It is an important surveillance in AMR (Anti-Microbial Resistance) with selective assessment approaches. In a clinical and surveillance setting, MIC determination represent a long-standing method of levels of resistance of bacterial population. Although MIC data makes its analysis non uniform and complex, MIC provides a unique and valuable insights on resistance patterns and adaptive resistance.

Time kills curve (TKC)

In vitro and in animal models, the anti-infective effects of antimicrobial agent can be studied using Time kill Curve method (TKC) which provides a detailed information on the different time course of anti-infective effect. To learn about detailed information regarding the time course of antibacterial effect or anti-infective effects in both animal and invitro models, the TKC has been used. The purpose of this analysis is to monitor the effect of different concentrations of an antimicrobial agent overtime pertaining to the phases or stages of the bacterial growth (such as lag, exponential, stationary phase). For antiseptic agents, TKC needs shorter time kill kinetics study with different methodology. Unlike MIC and MBC assay, TKC determines the speed of cidal activity [8].

TKC procedure

This procedure involves mixing of equal quantity of compound (drug to be tested) and broth with organisms on the plate which is the baseline and at different time intervals of 1hour, 2 hour and 4 hours the plate will be inoculated to be inoculated in anaerobic jar. After 48-72 hours, the colony count will be noted on the incubated plates.

Anti-biofilm assay

The term antibiofilm is proposed as a natural or induced process leading to reduction of bacterial biomass through the alteration of biofilm formation integrity and for quality. Various herbal extracts are being tested against human dental plaque to evaluate the effectiveness of plant extracts on planktonic and adherent microorganisms which generally are resistant otherwise. The antibacterial testing is complete by MBC and TKC along with MIC. For periodontal purpose, antibiofilm assay plays a vital role.

Need for anti-inflammatory test

During periodontitis, the inflammatory cytokines like IL-6 and MMPs host cells in response to periodontal pathogens is regarded as the major contribution to the local tissue destruction. Based on this, therapeutic measures to inhibit the production of these host molecules are of challenging interest in management of periodontitis. The host cells of immature immune system such as macrophages and monocytes are the important target for lipopolysaccharide (LPS) produced by periodontal pathogens which is responsible in the production of proinflammatory mediators (IL-6,8) and MMPs. The important role of multipotent IL-6 cytokine is to regulate immune response to periopathogens specially the osteoclast differentiation occurring in the periodontal disease and stimulating MMP secretion.

The proinflammatory cytokines such as TNFα, IL-1, IL-6 mediate the inflammatory response in the body. IL-1 has diverse actions like immunologic, physiologic and hematopoetic effect which stimulate production of other cytokines such as IL-6 and TNF-α.

MMPs are a group of newly endopeptides which functions as effectors of extra cellular matrix remodelling in physiological and pathological conditions. They are classified as collagenase (MMP-1, -8, -13 and -18), gelatinase (MMP-2 and -9) and stromalyins (MMP -3, -10 and -11) and other MMPs. Tissue inhibitors of metalloproteinase (TIMPs) are a family of four endogenous antagonist that bind to the catalytic site of MMPs. The MMP-2 and -9 (gelatinase A and B respectively) have played an important role in cardiovascular diseases and ischemic heart diseases. Various tests used to measure anti-inflammatory effect of any therapeutic are zymography, Membrane stabilization assay, Lipozxygenase inhibition and Protein denaturation inhibition. The main advantage of gel zymog-

raphy is that both latent and active forms of both MMP2 and MMP 9 are possible to be detected as low as 1nm without the need of costly reagent.

Zymography is known as an electrophoretic technique, commonly based on sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE), which contains a substrate copolymerized within the polyacrylamide gel matrix (e.g gelatin), for the detection of an enzymatic activity. Zymography is suitable for analysis of MMPs in complex biological fluids (serum, synovial fluid) and tissue extracts (heart, liver, kidney, spleen, etc.).

Why gel zymography as an anti-inflammatory test? While plasma concentrations of MMP-2 and MMP-9 have been measured in many previous studies by using commercially available enzyme immunoassays (ELISA), quantitative gel zymography is thought to be favourably compared with ELISA [9]. This opinion is based on the fact that gel zymography is a powerful tool allowing the detection of both the latent and active forms of both MMP-2 and MMP-9 in concentrations as low as 1 nM. Other anti-inflammatory tests are presented in table 1.

Test	Explanation
Membrane stabilization assay	Majority of the anti-inflammatory drugs stabilize the plasma membrane of mammalian erythrocyte and thereby inhibit the heat-induced and the hypotonicity-induced haemolysis. Membrane stabilization involves the process in which the integrity of the erythrocyte's membrane and lyosomal membrane is maintained by anti-inflammatory drugs by stabilizing the membrane. The stabilizing effect of the drugs on erythrocyte membrane may be due to a stabilizing effect of the drugs on certain proteins in the membrane [16].
Lipoxygenase inhibition	Lipoxygenase (LOX) is one enzyme that has a role in inflammation, especially in the biochemical processes of leukotrienes. Leukotrienes are the main regulator of allergic reactions and inflammation. Currently, lipoxygenase inhibitors become a potentially important agent that shows significant anti-inflammatory activity [17].
Protein denaturation inhibition	The denaturation of albumin protein leads to the formation of antigens which initiate type III hypersensitive reaction leading to inflammation. The autoantigen production in inflammation is due to denaturation of protein and several studies reveal that protein denaturation is one of the reasons for inflammation. Protein denaturation is a process in which proteins lose their tertiary structure and secondary structure by application of external stress or compound, such as strong acid or base, a concentrated inorganic salt, an organic solvent, or heat. Most biological proteins lose their biological function when denatured. Denaturation of tissue proteins is one of the well documented causes of inflammation [18].

Table 1: Anti-inflammatory tests.

Need for immunomodulatory tests

The protection of the body from foreign invaders is dealt by complex organisation of immune system comprising of white blood cells, antibodies and blood factors. Immune system simultaneously maintains self-tolerance along with body protection [10]. The natural or innate immunological mechanisms provide a short term first line defense or through highly specific, complex and diverse long term adaptive mechanisms with additional characteristic of memory.

The human immune system consisting of T cells, B cells, NK cells, eosinophills, basophils, neutrophils, mast cells, macrophages

and those mediators like cytokines interacts with non-immune cell types in a complex and dynamic network to extend protection to the body against external infective pathogens. In the course of tissue homeostasis disturbance during disease process, the mast cells and macrophages release soluble proinflammatory mediators like interleukins and TNF.

The different cell types elaborate cytokines which act in autocrine, paracrine or endocrine manner to regulate or simulate growth and function of neighbouring cells through specific receptors effectively in picromolar quantities to be highly potent. Cytokines are true language communication between cells of immune system. They are responsible for innate reaction by producing inflammation and being chemotactic.

The activity of immune system is modified by substances called immunomodulators. These substances may inhibit or enhance immunological response of an organism by interference in its regulatory mechanism. The nature of this antigen independent immunity is clearly different from the response achieved by passive immunization or by conventional immunization using antibodies [11]. The antigen production such as TNF, IL and interferons can be regulated by immunomodulators which in turn activate NK cells or T cells.

Various assays meant for immunomodulatory effect are Nitroblue Tetrazolium Assay (NBT) assays, Phagocytosis, Candidacidal assay and chemotaxis.

Phagocytes undergo NADPH oxidase catalyzed oxidative respiratory burst in response to various stimuli and as a result produce the superoxide anion (O_2) and hydrogen peroxide (H_2O_2). These reactive oxygen species serve as inflammatory mediators and play important roles in host defense by killing invading pathogens and they also induce a variety of antioxidant enzymes in host cells. The conventional microscopic NBT assay is used to qualitatively detect the O_2 produced in phagocytic cells. Other immunomodulatory tests are presented in table 2.

Need for antioxidant test

By activation of the host defense mechanism, the reactive oxygen species (ROS) or free radicals are formed by the action of periodontal pathogens and their product. The free radicals such as O_2^-, H_2O_2, HOCL, and OH- are highly reactive due to the presence of unpaired electrons. The activate PMNs release them to bring about oxidative killing of organism in biofilm which causes further release of proinflammatory mediators and cytokines (TNF-α, IL-1 α, β, IL-6 and IL-8) leading to amplified inflammatory process [12]. The prolonged ROS exposure leads to wide array of pathological reactions in host tissue (cell membrane lysis, fragmentation of DNA, enzyme inactivation, proteolytic enzyme activation leading to specific ECM degeneration of hyaluronic acid proteoglycans) [13]. The ROS release from phagocytes is detrimental to tissues in the surrounding vicinity which leads to pathogenesis of various inflammatory conditions by harmful oxidative reactions [12].

The antioxidant defense mechanism in the human body comprises of both enzymatic and non-enzymatic antioxidants. The oxidative injury brought by ROS generated by metabolic process are scavenged by antioxidant enzymes and the extend of inflammatory response is modulated. The super oxide dismutase (SOD), an intracellular enzyme protect the tissue cells from oxygen derived free radicals which causes superoxide anion dismutation to hydrogen peroxide and glutathione peroxidase. Catalase converts the H_2O_2 to oxygen and water. The antioxidant deficiency enhances oxidative stress resulting in tissue damage as a part of pathogenesis of various diseases.

The osteoblast recruitment and activity is said to be modulated by Nitric oxide (NO). As a response to oxidative damage, there is inhibition of osteoblast grow [14] and osteoblast apoptosis [15].

Various methods to test the antioxidant activity are 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging (ABTS) activity, Trolox equivalent antioxidant capacity (TEAC), Feric Reducing Antioxidant Power (FRAP). DPPH consists of dark colored crystalline powder made of stable free-radical molecules. It is the common antioxidant assay used, as it is a well known radical to scavenge other radicals. Hence, reduction rate of a chemical reaction up on DPPH addiction is used as an indicator of the radical nature of that reaction.

Few of the herbal extracts which are effective from the periodontal perspective are presented in table 3.

An *in vitro* study was conducted to compare the polyherbal gel and oleozone gel. (Diisertatn ref). The polyherbal gel (HiOra-SG)

Test	Explanation
Macrophage activity test	The assay can be adapted appropriately to test macrophage response to other agents as well that will be referred to herein as 'test reagents' or 'test compounds.
Carbon clearance test	Carbon clearance test evaluates the effect of drugs and phytoconstituents of the reticuloendothelial system (RES). RES comprises of a 'diffuse system' that make up of phagocytic cells. Once the colloidal carbon particles are directly injected into the blood, they are cleared by RES by the virtue of phagocytosis.
Mast cell degranulation assay	Our mast cell degranulation is a rapid assay that tests the ability of the test compound to induce mast cell degranulation and release of granule contents into the tissue culture after a short incubation time of 30 minutes
Polymorphonuclear leukocyte function test	The polymorphonuclear leukocyte PMN) represents the first line of defense against invasion by most bacteria and certain yeast and fungi. This cell must arrive at the site of microbial intrusion within a critical 2- to 4-hour period if infection is to be confined or suppressed. The acute inflammatory process by which this occurs is a complex series of events involving the complement system, bacterial products, and endogenous mediators. These affect the PMN, leading to a form of metabolic and functional "activation," and result in increased adherence, chemotaxis, phagocytosis, and heightened metabolic and bactericidal responses.
Neutrophil function test	The complexity of the neutrophil response to inflammation creates many difficulties for the study of neutrophil function in vitro. The environment in which a neutrophil is placed can have marked effects upon a variety of cellular functions. Quantitative tests of neutrophil function present problems not only with assay design but also in the isolation of cells from peripheral blood without disturbing their normal physiology. It is desirable to isolate neutrophils from other leukocytes because soluble factors released by other cells can influence neutrophil function, and other cells may interfere with functional assays; for example, monocytes will phagocytose opsonized particles and eosinophils contain a potent peroxidase. Attention to physical parameters such as temperature, pH or osmolarity, and rigorous exclusion of endotoxin, permits neutrophils to be isolated in a resting state. Subsequent function tests must be selected with an understanding of normal neutrophil physiology and applied with an awareness of any associated technical problems. The investigation of abnormal neutrophil responses may necessitate the screening of several tests of function; for example, defective neutrophil killing may be the result of abnormal chemotaxis, phagocytosis or degranulation [19].
Phagocytosis	Potential advantages of this assay are direct measurement of relevant biological activity, minimal sample processing and the potential to become a 'near-patient' test with sample to result time of less than 4h. It directly measures phagocytic association with, and oxidation of neutrophils (representative of host/pathogen interaction) and can deliver results within 4h of venepuncture.

Table 2: Immunomodulatory tests.

Author	Year	Aim	Conclusion
Clove			
Anti-inflammatory			
SJ Pulikottil., *et al*.	2015	Presented a comprehensive report on the properties of clove based on an analysis of contemporary scientific and professional literature in order to explore the prospects for its application in the treatment of plaque-induced periodontal diseases.	Clove and its derivatives have a definite potential to be used as specific anti-plaque and anti-inflammatory agents for the treatment of periodontal disease [20].
Ambarish S. Sindag., *et al*.	2020	To isolate, identify, and characterize the periodontal pathogens from infected patients and to check the antimicrobial activity of the isolated pathogen against neem, clove, and cinnamon extracts using agar well diffusion method	Results showed an effective zone of inhibition (24 and 22 mm) against Actinobacillus sp. at 10% concentration, while cinnamon aqueous extracts exhibited a moderate zone of inhibition (16 mm) at the same concentration suggesting that neem, clove, and cinnamon extracts can be explored as an alternative therapy in the treatment of chronic periodontal disease [21].
Immunomodulatory effect			
Bachiega TF., *et al*.	2012	To analyse the immunomodulatory/anti-inflammatory effect of clove and eugenol on cytokine production (interleukin (IL)-1β, IL-6 and IL-10) in vitro.	Clove exerted immunomodulatory/anti-inflammatory effects by inhibiting LPS action [22].
Antioxidant effect			
Amani., *et al*.	2013	Antioxidant, Antimicrobial Activities and Volatile Constituents of Clove Flower Buds Oil	Volatile oil showed strong antioxidant activity against DPPH as compared with vitamin E [23].
Jasmine			
Antioxidant			
M. Umamaheswari., *et al*.	2007	Evaluate the antiulcer and antioxidant activities of 70% ethanolic axtract of leaves of Jasminum grandiflorum L. (JGLE) using acetic acid induced (AC) chronic ulcer model in rats.	Leaves of Jasminum grandiflorum possess potential antiulcer activity, which may be attributed to its antioxidant mechanism of action [24].
Anti-inflammatory			
Yohannes Tadiwos., *et al*.	2017	A study aimed at investigating the analgesic and anti-inflammatory activity of 80% methanol extract of J.abyssinicum root	inhibited pain sensation in the pain models and demonstrated anti inflammatory effect in the inflammation models in mice [25].

Nidhi Sengar	2014	A study was done to scientifically validate anti-inflammatory, analgesic and anti-pyretic activities of roots from J. sambac in blood serum and edematous tissue of rats exposed to acute (carrageenan) and granulomatous tissue in sub-chronic (cotton pellet granuloma) inflammation models	anti-inflammatory, analgesic and anti-pyretic activity of EJS which may be attributed to the presence of various phytoconstituents quantified especially hesperidin which have already been reported for its significant role in treatment of inflammation and associated problems [26].
Licorice			
Antibacterial			
Vivek K. Gupta., *et al*.	2008	to investigate antimicrobial potential of Glycyrrhiza glabra roots	Use of licorice as antitubercular agent through systemic experiments and sophisticated anti-TB assay [27].
Bone preservation			
Eun-Mi Choi	2005	A study investigated the effects of glabridin on the function of osteoblastic MC3T3-E1 cells and also the TNF-a-induced apoptosis and production of PGE2 and nitric oxide (NO) in osteoblasts	The estrogenic plant product, glabridin, has a direct stimulatory effect on bone formation in cultured MC3T3-E1 osteoblast cells in vitro. Thereby proving glabridin as a useful tool in the prevention of osteoporosis [28].
Antiinflammatory effect			
YuFu., *et al*.	2013	Antioxidant and anti-inflammatory activities of six flavonoids separated from licorice	The antioxidant and/or anti-inflammatory activities of three flavonoids were reported [29].
Antioxidant			
Jacob Vayaa	1997	Analysed the antioxidative properties of natural compounds from the root of the plant Glycyrrhiza glabra (licorice) toward LDL oxidation.	Very potent antioxidants toward LDL oxidation with Glabridin being the most abundant and potent antioxidant [30].
Neem			
Antioxidant activity			
Fong LO., *et al*.	2014	Evaluated the antioxidant potential of aqueous leaf extracts of the neem tree by the Folin-Ciocalteu assay, and antioxidant activity by the phosphomolybdenum method.	Neem leaves are a rich source of phenolic compounds with antioxidant properties [31].
Anti-inflammatory			
Marc Schumacher., *et al*.	2011	Evaluated the anti-inflammatory, pro-apoptotic, and anti-proliferative effects of a methanolic neem (Azadirachta indica) leaf extract are mediated via modulation of the nuclear factor-**κB** pathway	Strong effect of the neem extract on pro-inflammatory cell signaling and apoptotic cell death mechanisms, contributing to a better understanding of the mechanisms triggered by Azadirachta indica [32].

Dr. Jagadeesh. K., *et al*.	2014	Evaluated the anti Inflammatory effect of Azadirachta Indica (Neem) in albino rats	Can be safely used as a potent anti-inflammatory agent [33].
Antibacterial			
Sateesh Chickkanayakanahalli Parashuramaiah, Thoidingjam Pretty Chanu	2019	Evaluated the effects of neem leaf and neem twig extract on Prevotella Intermedia and Fusobacterium Nucleatum	It can be concluded that four different concentration of neem leaf extract i.e., 5%, 7.5%, 10% and 15% were effective against the Prevotella Intermedia and FusobacteriumNucleatum [34].
Immunomodulatory			
S N Upadhyay., *et al*.	1992	Immunomodulatory effects of neem oil were studied in mice	Neem oil acts as a non-specific immunostimulant and that it selectively activates the cell-mediated immune (CMI) mechanisms to elicit an enhanced response to subsequent mitogenic or antigenic challenge [35].
Spreading hogweed			
Immunomodulatory effect			
Kanjoormana Aryan Manu., *et al*.	2009	To study on the Immunomodulatory activities of Punarnavine, an alkaloid from Boerhaavia diffusa using Balb/c mice.	These results indicate the immunomodulatory activity of Punarnavine [36].
Anti-inflammatory effect			
S.A, Kalasker V	2014	to evaluate anti-inflammatory activity of aqueous extract of Boerhaavia diffusa leaves.	Boerhaavia diffuse extract showed significant anti-inflammatory action in acute and sub-acute experimental models and the activity were dose dependent [37].
Antioxidant activity			
K. S. Shisode and B. M. Kareppa	2019	to evaluate the antioxidant activity and phytochemical constituents of Boerhaavia diffusa Linn. (Nyctaginaceae) dried roots.	ethanol extract has shows better antioxidant activity as compared to remaining two [38].
Vanatulsi			
Antimicrobial Action			
Manasa Hosamane	2014	Evaluated the antibacterial efficacy of holy basil in vitro against some periodontopathogens and its antiplaque effect in vivo.	the holy basil mouthwash has an antiplaque effect and is efficacious against P. intermedia and F. nucleatum strains in vitro. Hence holy basil mouthwash may have potential as an antiplaque mouthwash with prophylactic benefits [39].

Sajjanshetty Mallikarjun	2016	to assess the in vitro antimicrobial activity of Tulsi leaves extract (Ocimum sanctum) on periodontal pathogens	Tulsi demonstrated effective antimicrobial property against Aa, suggesting its possible use as an effective and affordable "adjunct" along with the standard care in the management of periodontal conditions [40].
Aloevera			
Antibacterial Action			
Mohammadmehdi Fani., *et al*.	2012	Inhibitory activity of Aloe vera gel on some clinically isolated cariogenic and periodontopathic bacteria using the disk diffusion and micro dilution method	Aloe vera gel at optimum concentration could be used as an antiseptic for prevention of dental caries and periodontal diseases [41].
Olive oil			
Anti microbial action			
Giampiero Pietrocola	2018	Evaluation of the antibacterial activity of a new ozonized olive oil against oral and periodontal pathogens and compared with that of common CHX-based agents using direct contact agar diffusion test (DCT) and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) evaluations	Gram-negative bacteria proved to be more sensitive to ozonized olive oil than Gram-positive ones. The ozonized olive oil demonstrated a lower antibacterial activity if compared to the CHX-based agents tested [42].
Ozone			
Antibacterial			
Sigrun Eick	2012	Determined the effect of ozone on periodontopathogenic microorganisms	ozone has a strong antibacterial activity against putative periodontopathogenic microorganisms, and the bactericidal effect is reduced in the presence of serum. Ozone may have potential as an adjunctive application to mechanical treatment in periodontitis patients [43].
Tatsuya Fukui., *et al*.	2014	Evaluated the antimicrobial effects of ozone gel against periodontal bacteria	the ozone gel can be clinically useful in oral surgery for implant treatment because of its instantaneous antimicrobial effects, and that it can be used against a variety of bacterial strains [44].

Table 3: Review of literature of herbal extract.

which consists of Jasminum grandiflorum (jati-20 mg), Glycyrrhiza glabra (yashtimahu-licorice-8 mg), Triphala (6 mg), Boerhaavia diffusa (punarnava-spreading hogweed- 6 mg), Syzygium aromaticum (lavanga-clove-5 mg), Ocimum basilicum (vanatulsi-3.3 mg), Azadirachta indica (nimba-neem- 1.7 mg) and ozonated olive oil gel with principle ingredients of Aloe Vera, olive oil and ozone are marketed to avail the best of the therapeutic benefits to treat oral, dental and periodontal diseases. Most often it is used in various branches of dentistry to treat oral ulcers, infectious candidial bacteria, dentinal hypersensitivity, bleeding gums and periodontitis conditions. Based on its composition and its available literature, its effectiveness on periodontopathogens has not been tried along with its anti-inflammatory, antioxidant and immunomodulatory effect. The invitro tests which include antibacterial activity, anti-inflammatory activity, immuomodulatory activity and antioxidant activity of this polyherbal gels has been studied for the first time and are discussed below.

Antibacterial activity

The antibacterial activity was tested against: Porphyromonas gingivalis (Pg), Prevotela intermedia (Pi) and Fusobacterium nucleatum (Fn). Both MIC and MBC were analysed at 100 µg/ml,50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.12 µg/ml,1.6 µg/ml,0.8 µg/ml,0.4 µg/ml and 0.2 µg/ml. TKC was determined at baseline,1 hour, 2 hour and 4hour.

Minimum Inhibitory concentration (MIC) test revealed that, Pg, Pi and Fn were resistant to HiOra-SG gel at 0.2 µg/ml. Whereas, Pg and Fn were resistant to Oleozone at 0.4 µg/ml and Pi at 0.8 µg/ml indicating that HiOra-SG gel had better MIC than Oleozone gel.

Minimum Bacterial Concentration (MBC) test revealed that, Pg, Pi and Fn showed growth against HiOra-SG gel at 0.2 µg/ml. Whereas, Fn and Pg showed growth against Oleozone gel at 0.4 µg/ml and Pi at 0.8 µg/ml indicating that HiOra-SG gel had better MBC than Oleozone gel.

Time kill Curve test

At baseline, HiOra-SG showed microbial activity of > 150 for Pi and Fn, > 200 for Pg and later at 1 hour,2 hour and 4 hour no growth was reported. Oleozone at baseline showed microbial activity of > 200 for Pi and Fn, > 350 for Pg but later at 1 hour showed activity against Pg (200) and Pi (150). After 2 hour and 4 hour no growth was reported indicating that HiOra-SG gel had better time kill curve than Oleozone gel.

Anti-inflammatory activity

The anti-inflammatory activity was tested against MMP 2 and MMP 9. Immunomodulatory activity of both test gels were assessed at 10,5 and 2.5 concentrations along with positive and negative control. Oleozone gel showed 85% activity against MMP-2 and 50% activity against MMP-9 while HiOra-SG gel showed 90% activity against MMP-2 and 80% activity against MMP-9. The results conclude that HiOra-SG gel showed better invitro anti-inflammatory activity when compared to Oleozone gel.

Immunomodulatory activity

HiOra-SG gel at three consecutive concentration of10,5,2.5, positive and negative control showed NBT as 22%, 12%,12%, 64% and 8% respectively; Phagocytosis at 4,4,3, 5 and 2 respectively; intercellular killing at 40%,30%,30%,10% and 0% respectively; chemotaxis at 1.2 mm, 0.8 mm, 0.5 mm, 2.4mm (FMLP) and 0.5mm (medium) respectively. Oleozone gel at three consecutive concentrations i.e., 10,5,2.5 and positive, negative control showed NBT as 12%, 8%,7%, 60% and 11% respectively; Phagocytosis at 2,2,2, 5+ and 1 respectively; intercellular killing at 18%,12%,12%,12% and 0% respectively; chemotaxis at 2 mm, 0.9 mm, 0.6 mm, 2.2mm (FMLP) and 0.5mm (medium) respectively.

Antioxidant activity

HiOra-SG showed 36.8% antioxidant. Whereas Oleozone didn't detect any antioxidant activity indicating that HiOra-SG gel has better antioxidant activity when compared to Oleozone gel.

From the invitro observations made from this study, HiOra- SG gel showed better antibacterial, anti-inflammatory, immunomodulatory and antioxidant activity against than Oleozone gel.

For a long time, various bacterial infections are treated by use of natural remedies. In the practice of bacterial resistance and intolerance are due to the misuse of antibiotics. The phytoscience resources, an evolving multidisplinary science, has explored to reveal several medicinal plants possessing antibacterial activity along with other therapeutic potential and minimum toxicity. The structurally different herbal phytochemicals than classical microbially derived antimicrobials provide a new choice for selective antimi-

crobial therapy against various oral microbes. Being distinct antimicrobial allopathic compounds, the microbial pathogens showed no development of resistance.

In both developed and developing countries. Medicinal plants are considered an important part of pharmacopeias as these plant extracts are more effective and freer from desirable effects.

There is limited literature available on the clinical, microbiological and biochemical estimation of clove, jasmine, spreading hogweed and vanatulsi which can be dealt in the further studies. Further studies can be directed by the use of herbal agents with multipotential activity effective against those pathogenic mechanism responsible for periodontitis initiation and continued destruction. There is a good scope for exploring various herbal agents from the glossary of herbal medicine by evaluating its invitro potential and to have a clinical activity with a safe and comfort zone.

Conclusion

Clinical limitations to the effectiveness of root planing and possible recolonization of disease associated bacteria have led to addition of antimicrobials both systemically and locally as adjuncts to mechanical therapy. Aloe vera, clove, Jasmine, Licorice, Neem, Olive, Ozone, spreading hogweed, Triphala and Vanatulsi are known to have anti-inflammatory, antibacterial, antioxidant, and immunomodulatory activity. However combination of the herbs and ozonated olive gel were studied for the first time in the literature for the treatment of periodontal diseases. Based on the *in vitro* analysis, both prepartions are recommended for clinical use.

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