7.0 SUMMARY

Traditional macrofungal ethnomedicinal practices of communities from Dang and Purna are documented and evaluated herein. This is for the first time that *Phellinus durissimus* and many other species have been reported for ethnomedicinal uses. The immunomodulatory, anti-inflammatory, antioxidant and cytotoxicity bioactivities discovered by this work is the 1st report ever for this species.

Preliminary informal field visits at Gujarat and interaction with local people delivered the impression that there may be a substantial quantum of macrofungal diversity possibly harnessing valuable traditional practices. Pursuing it further, few primary investigations were made to gauge the plausible size, quality, uniqueness and location of the macrofungal diversity in Gujarat. It covers the Western part of India and is surrounded by Pakistan and Rajasthan in the North, Madhya Pradesh in the East, Maharashtra in the South-East and Arabian Sea in the West. It encompasses various ecosystems like grasslands, dry and wet deciduous forests, deserts, scrublands, wetlands, mangroves, coral reefs, estuaries and gulfs. The total forest cover of Gujarat is around 14,946 sq km, of which 114 sq km is very dense forest. This scanty resource of the state is also facing environmental challenges. Due to which loss of habitat is most obvious along the face of passing time. Thus in order to study the man-mushroom-medicine interface a proper location was to be selected. This location should be able to harbor a massive diversity of macrofungi. Only then the forest dwellers will have interaction with them. From these anthropological interactions several traditional practices take birth.

In order to have a glimpse of the probable size of macrofungal biodiversity, a theoretical estimate was made. After Insects, fungi withhold the second largest biodiversity. The huge figure of total estimated species of fungi is 1.5 million. This theoretical estimate is based on the average ratio of fungi to flowering plants. On two such canons of ratio the estimate was simulated to give the liberal and conservative arbitrary figures of biodiversity. The higher ceiling came to around 3000 species and the lower conservative estimate stood around 1100 to 440 species of macrofungi. Out of these species possibly 800 to 160 species could be unique to Gujarat. This would mean that there is a sizeable sum of species.
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diversity and greater molecular diversity therein. Hence on theoretical grounds it was seemingly withholding several putative drug lead candidates.

Massive bioprospecting programs between several countries across the continents has been done, though it is not par potential owing to the size of whopping biodiversity. Bioprospecting is the purposeful evaluation of wild biological material in search of valuable new products. In this regard ants of the tribe Attini and other termites known to rear and consume mushrooms for over 50 million years are the natural frontiers of bioprospecting. But mankind was not behind as since time immemorial we have been using natural resources for the betterment of the society. Bioprospecting can be done in many aspects. Since the inception of the very term it has been associated with drug discovery. This work is totally aligned on this track but on a smaller scale. Hence the present work was called **micro-level bioprospecting** as a limited number of species were documented within a restricted location. Reverse pharmacology led identification of active fraction and pharmacophore was the intended frame of research.

Out of the several species growing in the wild it would unwise to decide intuitively as all of them have their ecological expertise for the inhabited niche. Thus a concrete yard-stick was needed to bring about tangible outputs. The ethnomedicinal practices were just perfect to roll the matter ahead. Thus based on the idea that several unique species are there, their ethnobotanical uses were to be documented. In this regard due-diligence was focused on three aspects the location, possibly rich biodiversity and the community. The location was decided with respect to the densest forest area. The hypothesis behind this is as the denser forest area will harbor greater plants species in number and diversity, the diversity of macrofungi will thus be larger and richer, which in turn increases the probability of man mushroom interaction. To confirm the hypothesis we allocated a positive control region with opposite climate and ecology. The denser region was the Dangs in South Gujarat and the rarer one was Jessore in North Gujarat. Moreover, several tribal community in and around the Dangs were found to possess rich knowledge about medicinal plants. This was in line with the hypothesis of greater diversity and more ethnomedicinal practices. The research suffered due to lesser number of authentic traditional practitioners, and lower number of macrofungal practices in comparison to the plants.
Unlike *mega-bioprospecting* programs that screen thousands of plants, the *micro-level bioprospecting* was aimed at validating the practice, conserving important species, counter traditional knowledge dilution, generate scope for production of botanicals from the active fractions of the species employed and address local malnutrition and related health issues. Thus *micro-level bioprospecting* is by and from the local traditional knowledge practitioners and for welfare of the same. Thus it emanates and loops back to the local-level first. Later on as the research transpires to the next level as drug-discovery, equitable sharing of the benefits by the practitioners or the community can be appreciated. Hence, in such kind of models chance of biopiracy in almost nullified. This is possible when the investigational stake-holder has a neutral stand towards the know-how contributors and end-beneficiaries. In a nutshell *micro-level bioprospecting* is thus not just about smaller researches or surveys, but pertains rather to the providers as the immediate beneficiaries.

Putting all these pieces together the documentation of the Traditional-ethnomyco-medicinal practices (TEMPs) were done. The collection of materials was done with prior permission of the Department of Forest, and the documentation of which are endorsed under the Biodiversity Act. The samples were identified with the help of literature. The TEMPs were documented from Jessore Community (*JC*) and Purna Community (*PC*) from the North (Scarcer forest region) and South (denser forest region) Gujarat respectively. Of 20 informants consented in each community (total 40) 15 were short-listed and interviewed. Those presenting vague or ambiguous practices were excluded. For the sake of homogeneity of the study the types of informants were equi-proportional between both the communities. Of total 15 candidates of a community, 3 were traditional medicine practitioners, 10 were farmers and 2 were housewives. The TEMPs were observed to be widely different between both the communities. The types of species also varied with respect to their taxonomic origin and ecological niches. The traditional ethnomycomedicinal practices revealed around 23 total species which were recognized based on the rigorous cross-checking of answers to the questionnaires. The number of TEMPs found in JC was much lesser to that in PC. The JC reported 5 TEMPs in comparison to the 18 TEMPs from PC. This 23 macrofungal species differed in taxa, ecology, TEMPs, and specificity of application.
Nine species were used to treat general aspects like convalescence, whereas others (14 species) were used for specific ailments. In the Jessore community, 5 of the 7 ailments (71.2%) were related to skin problems, whereas in Purna community only 7 of 18 ailments (38.9%) had similar usage.

Even this sum of species was not approachable as each and every TEMP can’t be validates. Moreover all of them may not even deserve so, as some practices were very general. Herein we further bifurcated bioprospecting into wet and dry approaches. Since \textit{micro-level bioprospecting} is multifocused and is beyond singular aims like drug discovery, a harmony between all the requirements and objective were to be maintained. In order to further narrow-down the ethnomedicinal inventory a \textit{dry bioprospecting} was engaged. The aim was to first segregate the practices according to their characteristic features and assess the quality of data documented. This was achieved at several levels that evolved after each round of data analysis. The \textit{dry bioprospecting} was initialized by validating the data documented by consensus analysis of TEMPs in terms of Factor of informant consensus ($F_{ic}$), which tests the reliability, homogeneity and the extent of selection of certain species for treating an ailment. The total ailments addressed and species documented from the Purna community had a greater diversity and bore higher informant’s consensus index value in comparison to the Jessore community. The values were high for most of the ailments ranging from 0.9 to 0.928, except three ailments, which gave erroneous results due to only 1 report.

To classify the types of species according to TEMPs, binary scores were allotted based on the presence or absence of species storage for later use, open sharing and specificity of the practices. Followed by this species scores were subjected to Euclidean distance dissimilarity matrix based hierarchical agglomerative clustering. According to this analysis two major clusters were formed. Certain corner-stone species with closed (absence of) knowledge sharing were found to withhold specific practices. Those with general TEMPs as use for convalescence can be useful as nutraceuticals, whereas those with specific TEMPs can be screened further in order to identify pharmaceutical or nutriceutical potentials.

Of several macrofungi documented to have traditional uses few that had non-specific practices or not addressing any specific ailment were separated.
Nine such mushrooms formed the group of species that were investigated for potential regarding nutraceutical, functional food and dietary aspects in terms of their nutritional composition. Presence of phenolic components suggested that they may have considerable amount of antioxidant activity. The proximate analysis of the preliminary nutritional profile showed that most of the species had substantial level of carbon, nitrogen, phosphorus and niacin levels. Overall, the mushroom tagged TV based on the local name, surfaced as a potent species, which can be essential in combating malnutritional problems in the very tribal region it was collected from. It was found to contain around 66% carbohydrate and 27% protein. The state of Gujarat is reported to face about 61% of mortality rate in children below five due to severe malnutrition. Moreover the severity of acute and chronic protein energy malnutrition is reported to be very high in Gujarati children. They are stunted or wasted, or stunted and wasted, or stunted and obese. Over and above Gujarati adults are said to be thin with low protein and fat reserves partly due to protein energy malnutrition. It was also found to be at par with the nutritional levels of various other wild edible species around the world. It was then identified taxonomically as *Lentinus squarrosulus* Mont. During the documentation of practices, some subjects had mentioned about stomach discomfort if it was over eaten or eaten with stipe. In addition to this the subjects connected this problem when they consumed from broad leaf trees rather than those growing at the base of bamboos. On further investigation we found that the species that grew on tree trunks was not *Lentinus squarrosulus* but some other species of *Lentinus*. The second problematic species had visual resemblance because of which the tribals identify both as TV. Thus to test the hypothesis that *Lentinus squarrosulus* is not toxic and counter check its efficacy the samples collected from forest were subjected to two week acute oral toxicity testing. On evaluation in male and female SD rats after a single oral dose of 500, 1000 and 1500 mg/kg body weight, no effect on body weight was observed. In addition to this neither any major clinical signs were found nor were any abnormalities detected during necropsy. This rendered that the species was safe and can be pursued for further research. Thus it can be used against protein energy malnutrition and other types of malnutrition. Moreover local people can grow it throughout the year, to reap commercial benefits.

On the other hands the cluster of 14 species, had specific TEMPs to their name were to be further narrowed down. These were *Termitomyces tyleranus*,
Xylaria sp., Coprinus comatus, Bovista sp., Phallus sp., Lepiota cristata, Dictiophora sp., Scleroderma sp., Phellinus durissimus, Phellinus linteus, Phellinus merrillii, Phellinus rudis, Phellinus robiae and an unidentified species (Rd). Traditional Ethno-Mycomedicinal Practice Lattice Evaluation (TEMPLE) of these species revealed that Purna community was found to be richer in traditional know-how. This fell in place with our initial hypothesis of greater the biodiversity more and better the TEMPs. Hence selection of Dang as a potent geographic location proved to be valid. The Jessore community depicted diseases like pneumonia, constipation, eczema, which were high scorers with bruised skin infection, lesion, boils and wounds, scoring its half. Whereas Purna Community exposed practices for therapeutic potentials like immunostimulation (prophylaxis), or diseases such as boils, wounds, rashes, lesions (psoriatic), pimples, impetigo, pustules and fissure. Identification of the working group that contained several species of Phellinus and an unidentified species Rd was done on the basis of multivariate analysis elaborated below.

From this working group the isolation of the prime contender was to be done in line with the potent activity and a set of decision making factors. This was divided in to various indices that were scored and cumulatively calculated. Traditional Ethno-Mycomedicinal Practice Lattice Evaluation Score (TEMPLES) contained Activity Index, which was calculated from Core and shell sub-indices, and Non-activity index estimated from Decisive and Critical subindices. The activity index took in account various anti-inflammatory (AII), antimicrobial (AMI), immunostimulatory (MSI) and wound healing Indices (WHI), that were scored by multiple facets of treatment or ailments. Co-species priority index (CPI) is used to highlight importance of one species over another. Mode of Application Index (MAI) stressed upon the mode of application or consumption. Economic index (EI) is adapted to the necessary modifications needed for scoring those practitioners who rendered services for free or left it to the discretion of the beneficiaries, which had to be scored lower than those done only in lieu of prime economic favors. Feasibility index (FI) addresses the factors that are to dealt with practically. Knowledge Transfer index (KTI) assessed the kind to TEMPs that were practiced within a pool or in general. Dereplication from literature index (DLI) is an index to refurbish the TEMP by its present status with respect to the amount of work done based on literature review. Community knowledge richness index (CKR) of TEMP
from Jessore Community (JC) or Purna Community (PC) was added respectively to imbibe the integrity of community know-how level.

The activity group formed the crux of the study as it addresses the pharmacological activity of the species underlying the TEMP, rest of the indices were employed to rationalize a doable task within the given frame of resources and objectives. The scores of each species were subjected to multivariate analysis like hierarchical agglomerative clustering in 2D, principal component analysis and multidimensional scaling. TEMPLES revealed Rd and *P. durissimus* as the high scorers, of which the former was highest owing to a particular index. Considering the underlying factors behind the score of a species rather than direct numerical interpretation *P. durissimus* was isolated from the pool of working group as the prime contender based on overall credentials. Hereafter the true wet-bioprospecting was initiated by engaging a battery of pharmacological assays lineup with the plausible rationales of bioactivity.

*P. durissimus* is used by the tribal people to treat mumps and some other ailments in absence of which other species of *Phellinus* like *P. linteus*, *P. merrillii*, *P. robineae* and *P. rudis* are used. Probable immunostimulation induced by *P. durissimus* seems to be instrumental in subduing the mumps-induced immunosuppression. Thus the extracts and fractions of *P. durissimus* were examined against a battery of relevant *in vivo* immunomodulatory assays, in order to validate the traditional claims and characterize their dissimilar influences on innate and adaptive immune response. The methanol (*PdM*) and water (*PdW*) extracts against *in vivo* immunomodulatory assays addressing innate and adaptive immune responses (100, 300 and 600 mg/kg) were done through carbon clearance, haemagglutinating titre, spleen antibody forming cell and delayed type hypersensitivity assays. PdW and PdM showed better stimulations for innate and adaptive immune response at 300 mg/kg. In *Eschericia coli* induced bacteraemia model PdM alleviated mortality (65% survival) and showed greater inhibition of bacteraemia (88.5% INB) than those treated by PdW (50% survival, 5.16% INB). PdM was then partitioned into ethyl acetate soluble (*PdEs*) and insoluble (*PdEi*) fractions for further evaluation (300 mg/kg). PdEs outperformed its sister fraction and at times even its parent extract in enhancing adaptive immune response. PdEs afforded balance between reduction (50%) in mortality and bacterial clearance (85.4%), whereas PdEi had largely reduced mortality (75%) without any significant bacterial clearance (11.73%). In addition to this it showed significant *in
vivo proliferation of splenocytes and thymocytes, which may be related to increased IL-2 secretion. The active fraction PdEs showed good stimulatory index, which was greater for in vitro rather than in vivo indicating that it may be rendered labile during metabolism. Apart from this, it afforded reversal of cyclophosphamide mediated immunosuppression. Thus the PdEs can be deduced to possess potent immunostimulatory activity. This is the first report of the pharmacological activity of *P. durissimus* and delineates the immunomodulatory activity in accordance to the local traditional practice.

Thus it can be asserted that PdW had greater stimulation of the non-specific immune response, whereas PdM, PdEs, and PdEi gave better stimulation of specific immune response. Of both the fractions, PdEs gave better results in most of the assays targeting specific immune response, supported by mediocre decrease in mortality in *E. coli* induced peritonitis and bacteraemia model. This also suggested that PdEs can play an important role as an immunostimulant whereas PdEi and PdW may be involved in down regulating the extreme effect of immune response possibly by inducing anti-inflammatory cytokines. PdEs was found to have immunosuppressive activity that may be exerted directly or indirectly through various immune-pathways. It has significant effect on immunosupression of neutrophils and lymphocytes. It is also effective in reversing cyclophosphamide mediated suppression.

Apart from curing mumps, *P. durissimus* was also found to effectively reduce swelling of the parotid glands, commonly inflamed during mumps. Thus there could be possible antiinflammatory activity as an underlying action that renders it ethnomedicianly active. PdM afforded 59.48% (300 mg/kg) inhibition of edema. This was greater than PdW (49.24%-600 mg/kg), but lesser than indomethacin (74.29%-10 mg/kg). The ethyl acetate soluble (PdEs) and insoluble fractions (PdEi), which inhibited edema by 66.41% and 49.09%. Both these fractions (300 mg/kg) and PdW (600 mg/kg) were further assessed by histamine-and formalin-induced paw edema, acetic acid-induced vascular permeability and cotton pellet-induced granuloma assays. Though lesser than PdEs, yet PdW and PdEi had significant effect on the latter phase of carrageenan-induced edema, moderate effect on histamine-induced edema and marginal activity in formalin and cotton pellet chronic inflammatory models. Moreover, the reduction in vascular permeability and leukocyte infiltration exhibited by PdW and PdEi was comparable to or greater than PdEs. Further PdEs also exhibited significant anti-COX activity
that was in harmony with paw edema and other assays. The plausible anti-COX-1 activity of PdEs against uterus and erythrocyte membrane is one of the mode of actions by which it may exercise antiinflammatory activity. PdEi and PdW showed weaker effect on latter part of prostaglandin mediated phase but considerable reduction in vascular permeability and leukocyte infiltration.

_Phellinus durissimus_ is a plant parasite, and needless to say should overcome the host immune system to manifest itself. The plant immune system is known to produce damaging radicals which are to be encountered by the fungi, most probably accomplished by engaging an arsenal of chemical machinery that can also be useful as therapeutic antioxidants in combination to other possible activities. Salient _in vitro_ chemical assays like, DPPH (1, 1-diphenyl-2-picryl hydrazyl) free radical scavenging activity, superoxide radical scavenging activity, hydroxyl radical scavenging activity, lipid peroxidation and nitric oxide scavenging activity were employed. Apart from this, its capacity to face _in vitro_ biological assays was also studied employing rat liver lipid peroxidation assay and erythrocyte membrane stabilizing activity. Initially, the methanol extract (PdM) of the polypore was studied. Later the ethyl acetate soluble (PdEs) and ethyl acetate insoluble (PdEi) fractions of PdM were also studied for DPPH free radical scavenging activity, linoleic acid - ferric thiocynate and β-carotene bleaching test aiming at characterizing the kinds of activity differentiating amongst the two fractions. PdM showed considerable antioxidant activity in most of the assays, yielding IC$_{50}$ values between 50 to 100 µg/ml, baring hydroxyl radical scavenging activity and erythrocyte hemolysis test of which the former was much lower and later was higher than the rest. PdEs and PdEi showed substantial activity but performed best in either of the DPPH or lipid peroxidation assays. Phenols, flavanoids, anthraquinones and terpinoids were also found to be present, which may contribute to such kind of activity. In a nutshell PdM was found to be most active in all the assays in following order of decreasing activity, hydroxyl radical scavenging activity > linoleic acid - ferric thiocynate method > superoxide radical scavenging activity > DPPH free radical scavenging activity > rat liver lipid peroxidation assay > Nitric oxide scavenging activity > erythrocyte membrane stabilizing activity > β-Carotene bleaching test. In most of the assays with IC$_{50}$ of PdM was mostly below 100 µg/ml, suggesting that it has significant antioxidant activity. Of both of its daughter fractions PdEs demonstrated excellent DPPH free radical scavenging activity, whereas PdEi performed better in β-carotene.
bleaching and linoleic acid - ferric thiocynate assay. This implies that ethyl acetate soluble fraction of methanolic extract was not better than PdEi in arresting lipid peroxidation, though both showed active antioxidant potential. The level of performance was found to be better than other reports of mushrooms of the same or allied genus, in most of the assays. This also seems to profusely influence the immunomodulatory and anti-inflammatory activities of this species. This is the first report on the study of antioxidant potential of *Phellinus durissimus* that establishes it as a strong contender.

Several schools of traditional medicines advocate synergistic therapeutic effects (Gilani and Rahman, 2005), anticancer and immunostimulatory effects are one of such closely coupled synergisms. To assess the cytotoxic potential the hatchability assay of brine shrimp (HABS) was engaged. It delivered median hatch inhibitory concentration (HIC\(_{50}\)) of 62.31 for PdEs which was the most effective treatment, producing cytotoxicity. The brine shrimp cytotoxicity assay (BSCA) furnishing LC\(_{50}\) of 446 ng/ml for PdEs as per the contemporary assay methods. Extended lethality assay of brine shrimps (eLABS) devised by us, highlighted phased differential mortality with respect to growth. In addition to this the reduction in life span delineated by mean survival time (MST) explicated cytotoxicity better than LC\(_{50}\) alone and characterized the extracts and fractions at various doses, more efficiently. In order to get an essence of the kind of cytotoxicity the test fraction had, it was compared to standard cytotoxic agents such as doxorubicin, colchicine, cyclophosphamide, podophyllotoxin, 1,8 dihydroxy anthraquinone, chloroplatinic acid, potassium dichromate and gallic acid. For most of the concentrations PdEs gave very less mean survival time around 14 h. This closely resembled the activity of cyclophosphamide, podophyllotoxin, colchicine and lower concentrations of potassium dichromate.

To match the change in mortality along different concentrations during different periods of time corresponding to various phases of shrimp’s growth the mortality was clustered against time by 2D agglomerative higherarchical clustering. Herein PdEs showed a pattern of cytotoxicity that is closely related to cyclophosphamide, podophyllotoxin and colchicine. Thus it may be active even at very low doses.

PdEs thus surfaced as the most active fraction, which outperformed PdW and PdEi in most of the models of acute and chronic inflammation. By HPTLC analysis it was found to contain hispolon, coumarin and scopoletin, of which
The immunomodulatory potential of PdEs can be exploited against cancer combination therapy to potentiate the activity of cytotoxic drugs and as small molecule adjuvants to amplify the effects of subunit vaccines. Though the former needs clinical confirmation, it can be expected that PdEs will have assist homeostasis in immune cell counts of patients suffering from chemotherapy related cytopenia. Further fractionation is needed to segregate direct cytotoxicity and indirect cytotoxicity by immunopotentiation. The desired combination of cytotoxicity loaded with immunopotentiation, may be instrumental in conjunction with cancer vaccines. Considerable antiinflammatory activity in terms may lead towards preparation of formulations for arthritic and other inflammations. Its antioxidant potential can be exploited for nutra- or nutria-ceutical products and value addition in food industry as well.

*Phellinus durissimus* was hence validated to possess several potent bioactivities that needs further exploration. Thus each of two species identified from respective clusters of specific and non-specific practices can be used to address local micro-level issues as malnutrition, natural drug therapy and opportunities for revenue generation.