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Piperine production from endophytic fungi of *Piper nigrum l* and its *In Silico* approach for anti-inflammatory and anti-mycobacterial potential

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Abstract

Present study was aimed at culturing endophytic fungi Colletotrichum gloeosporioides from Piper nigrum L. and extracting alkaloid piperine from submerged fermentation of isolated fungal endophyte. We also aimed for an in silico approach on anti-mycobacterial and anti-inflammatory role of piperine. Stems of Piper nigrum L. were spread plated on fungal selective media (Arginine glycerol media; AGA), and hyphae from identified fungal culture were subjected for submerged fermentation in Martin's Rose Bengal Broth media. The secondary metabolite produced exogenously on broth media was extracted several times with ethyl acetate and concentrated by rotary evaporator. Screening was done via comparative study of crude extract and standard piperine under Thin Layer Chromatography (TLC) analysis. Piperine and some major derivatives were autodocked against active site of efflux protein Rv1258c of Mycobacterium tuberculosis. Comparative docking of piperine and some widely used NSAIDS with active site of COX2 enzymes was done to understand anti-inflammatory potential of piperine. Fungal endophytes identification was done on morphological basis by Lacto phenol Cotton Blue staining focusing on conidiophores and conidia. Crude extract from fungal fermentation and standard piperine showed similar TLC analysis and antimicrobial activity results. Auto-docking result showed piperitine to have least binding energy (-104.914kCal/Mole) with active site of efflux protein Rv1258c. Also, compared to widely used NSAIDs (Aspirin, Ibuprofen, Declofenac), piperine showed lesser binding energy (-92.383kCal/Mole) with active site of prostaglandin synthesizing enzyme COX2. Fungal fermentation mediated successful extraction of piperine in this study suggests for future approach for large-scale commercial production of such natural bioactive compounds. Piperine derivative piperitine can be successfully used in ant-tubercular therapy for enhancing bioavailability of antimycobacterial drug like rifampicin. The latent toxicity of anti-inflammatory drugs can be well addressed by use of natural bioactive compounds like piperine showing anti-inflammatory activity.

Keywords: Piperine, *Colletotrichum gloeosporioiedes, Piper nigrum* L., Rv1258c, COX2.

Introduction

Endophytes represent microorganisms that colonize asymptomatically inside healthy tissue of host plant and maintain mutualistic association for whole or part of their lifecycle[1]. De Bary, for the first time in 1986 used the term endophyte to describe the presence of non- pathogenic organisms inside the plant. Now it has been discovered thoroughly to embrace broad categories of microbes like fungi, bacteria, and actinomyces[2]. Clear evidence indicating co-evolution of plant and fungi came from fossilized fungal hyphae and spores (from Ordovician of Wisconsin, 460 million years ago), showing the role of fungal association with plant facilitating first colonization of land by plants[3]. Plants take advantage of endophyte colonization to survive in highly competitive ecological niches. Wide varieties of plants (about 300,000) are estimated to inhabit the endophytes. Study behind asymptomatic existence of endophytic fungi suggested that the endophyte overcomes the defense reaction from the plant host only up to threshold level, which enables them to be in harmonic relation with host plant. It has been referred as the balanced antagonism[4]. In order to make the stable association with the host, endophytes synthesize various bioactive compounds that support growth of host plant by making them able to resist external biotic and abiotic compounds. Endophytic fungi are therefore frequently reported as resources for plant related natural bioactive compounds[5]. The first ever observation made was of endophytic fungi producing paclitaxol; isolated from phloem of pacific yew tree, *Taxus brevifolia*. Several other bioactive compounds being produced are paclitaxel, podophyllotoxin, camptothecine, vinblastine, hypericin and diosgenin[6].

The identification of endophytic fungi and associated biosynthesis of bioactive compounds have been undoubtedly proven advantageous in many ways. With low expenses fungal culture media, it can be taken to large-scale production. There always scopes of media optimization for yield maximization and slight pathway modification on endophytes for producing similar compounds. Since endophytes are producing same

bioactive compounds as that of host plant, it reduces the need to harvest slow growing and possibly rare plants to preserve worlds' diminishing biodiversity[5,7].

Piper nigrum L. (Black pepper); well known as king of the spices, is the climbing type perennial flowering vine of *Piperaceae* family, bearing the drupes of peppercorn, cultivated for its spiciness and medicinal values. Numerous biologically important phytochemicals like alkaloids, amides, propenyphenols, lignans, neolignans, terpenes, steroid etc have been extracted from *Piper nigrum*. The major demonstrated phytochemical components are Piperine, Piperamine, Sarmentosine, Sarmentine, Trichostachine, etc[8]. Piperine (1-piperoylpiperidine) the primary alkaloid in black peppercorns is responsible for the pungency and this sensation is caused by heat and acidity sensing Transient receptor potential vanilloid (TRPV) ion channel on heat and capsaicin receptor TRPV1[9]. The pharmacological significances of piper nigrum are well illustrated by use of their blend as an ingredients in around 60% of ayurveda medicine and this is attributed majorly by the principle component; piperine. It shows wide range of properties ranging from antibacterial, antifungal, anti-inflammatory, antioxidant, anticancer, immunomodulatory, and bioavailability enhancing effect[10-12]. Moreover piperine shows carminative effect, causes reflex flow of saliva, increases secretion of gastric juices thus directing towards increased digestion and improved appetite[13].

Current study deals with isolation of piperine producing endophytic fungi *Colletotrichum gloeosporioides* from stem part of *Piper nigrum L.* From the *in silico* study, we tried to evaluate piperine and other derivatives as potential bioavailability enhancer of anti-mycobacterial drugs. We concluded piperitine as most promising candidate amongst all. Docking study of piperine and some of widely used anti-inflammatory drugs: Aspirin, Ibuprofen, Declofenac with active site of enzymes Cox1 and Cox2 responsible for prostaglandin synthesis. It suggested piperine to have least binding energy and therefore it could be strong alternative of the widely used chemical drugs. We found piperine as a strong candidate in search for novel source of medicinal compounds. Being fungal endophyte secondary metabolites, it ensures no toxicity to host plant as well as the animal system. Hence, it equally addresses problem of latent toxicity linked with synthesized chemical drugs.

Materials and methods

Fungal endophytes isolation: Stem pieces of healthy and mature *Piper nigrum* plants; obtained from Institute of Ayurveda and Integrative Medicine, Yelahanka, Bengaluru were used for isolation of endophytic fungi. Surface sterilization of plant materials was done under laminar flow hood following the steps described by Chithra et al[7]. Firstly, plant materials were washed with sterile distilled water for several times and immersed in $HgCl_2$ for 1 min. It was then followed by treatment with 70 % ethanol for 1 min and rinsing with distilled water for several times. The stem part was aseptically cut into several small pieces of mm size using sterile forceps and scalpel. Cutting was made first in transverse plane and then vertically. The cut parts were then spread plated on arginine glycerol agar media (composition (g/L): 20 glycerol; 2.5 l-arginine; 1 NaCl; 0.1 $CaCO_3$; 0.1 $FeSO_4 \cdot 7H_2O$; 0.1 $MgSO_4 \cdot 7H_2O$; 20 agar) with 50µg/ml nalidixic acid supplement (Figure-1A). In some plates, stem pieces were plated as it is i.e. without vertical cutting, which was used as negative control. Plates were then incubated at room temperature for 5 days for the fungal growth. The plates from where fungal isolates were identified were further maintained in MRBA media (composition (g/l): 10g glucose; 5g peptone; 0.5g K_2HPO_4; 0.5g KH_2O_4; 0.5g $MgSO_4.7H_20$; 0.5g Yeast extract; 20g Agar; rose bengal, pinch).

Morphological identification of fungal isolate: The mount preparation of Lacto Phenol Cotton Blue (LPCB) was used for observing fungi especially emphasizing on conidiophores and scattered conidia. One-two drops of lactophenol cotton blue stains were placed on microscopic slide on which fungal hyphae were teased to very fine strands. Then cover slip was gently applied over it avoiding air bubbles formation and observed under 40X objective. Spore morphology, vegetative structures, and hyphae growth pattern on plates were focused as parameters for identification of Colletotrichum.

Piperine production via submerged fungal fermentation and screening by Thin Layer Chromatography: Fungal cultures, identified as of Colletotrichum genus were further sub cultured on Martin's Rose Bengal Agar (MRBA) plates (Figure-1C). The non sporulated hyphae of such culture was transferred initially on 100ml broth of same media as an inoculum enrichment followed by transfer of vegetative state of fully grown culture to next 250 ml MRBB media; which was then incubated in shaking incubator at 30ºC for 30 days (Figure-2A). This was followed by filtering of fermentation broth using muslin cloth for separation of fungal biomass. Extraction of culture filtrate was done twice with one third volume of ethyl acetate and organic phase was concentrated using rotary evaporator at 50ºC under vacuum to collect crude extract powder which was resuspended in small volume of ethyl acetate for further screening steps. Methanol: water (70:30) was used as mobile phase to run the crude extract sample and standard piperine on Thin layer silica gel plates as a primary screening for presence of piperine by Retention factors (Rf) comparison.

Antimicrobial study: Both crude extract and standard piperine were implied for antimicrobial (antibacterial and antifungal) test by agar well diffusion method. Muller Hinton agar plates were prepared and swab cultured with the broth inoculums of *Escherichia coli, Pseudomonas putida, Staphylococcus aureus* and, *Bacillus subtilis.* Using cork borer, the wells were punched on agar plates and four wells were made per plate. Crude extract, standard piperine solution (50µg/ml) were added on two wells along with ethyl acetate as negative control and Streptomycin as the standard antibiotic on remaining wells. It

was followed by incubation at 37ºC, 24 hours. Zones of inhibition representing the susceptibility of bacteria were noted down. Antifungal test was done against *Aspergillus niger* and *Saccharomyces cerevisiae*. Inoculum of each fungus was swab cultured on MRBA media and after the full growth of fungi; wells were punched in similar manner. Crude extract, standard piperine, and organic solvent (ethyl acetate) were added to the wells and incubated at room temperature for one week.

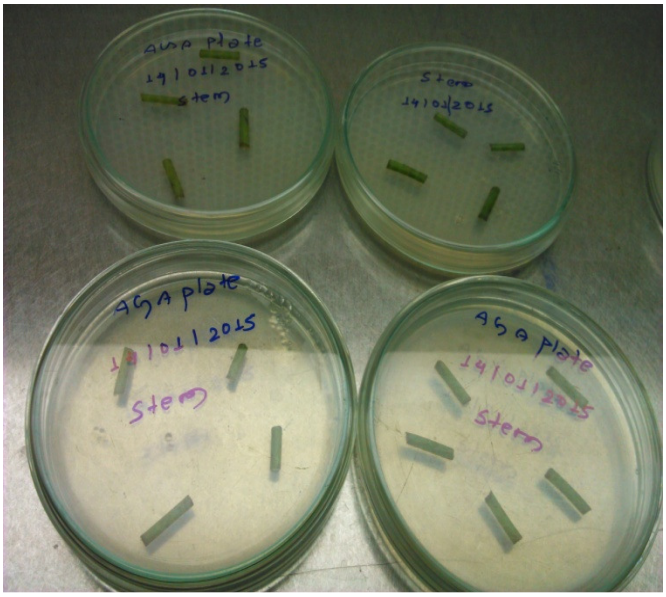


Figure-1A: Vertical stem cuttings were place on solid nutrient agar media with cut part facing on agar surface and incubated at room temperature for seven days.

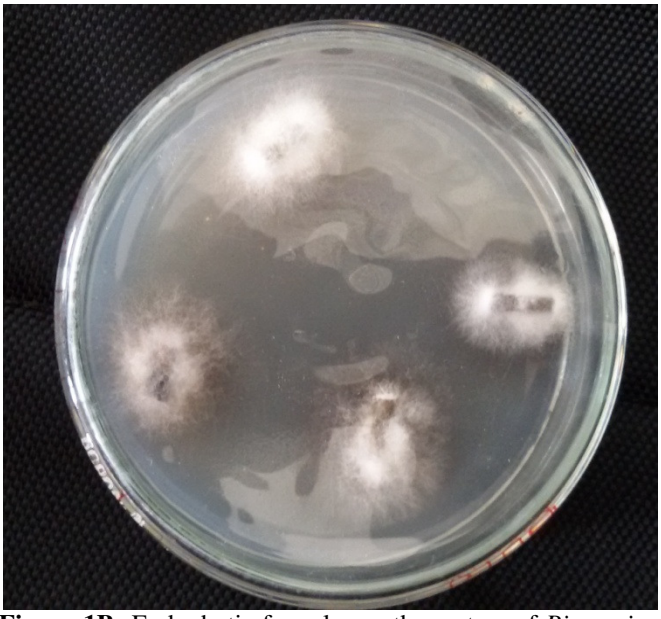


Figure-1B: Endophytic fungal growth on stem of *Piper nigrum L* was observed with whitish mycelium having heavily intertwining hyphae.

Figure-1C: The identified Colletotrichum hyphae subcultured on Martin's Rose Bengal Agar (MRBA) plates.

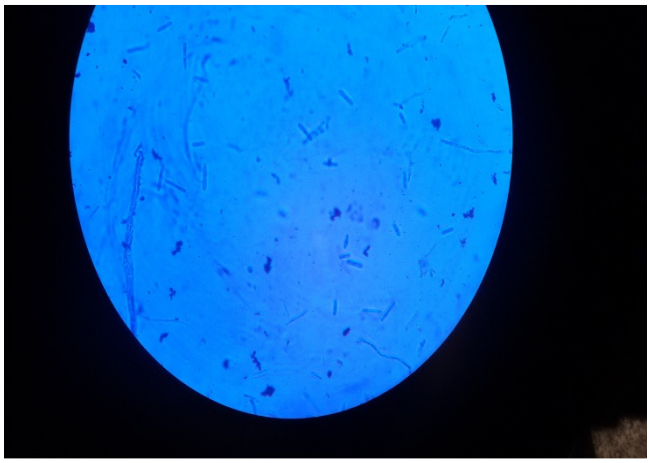


Figure-1D: Microscopic observation of conidia in isolated endophytic fungi after staining with Lactophenol cotton blue.

***In silico* screening of piperine for antimycobacterial and anti-inflammatory activity:** The inhibitors of efflux protein Rv1258c in *Mycobacterium tuberculosis* can result maximum bioavailability of anti-tubercular drugs. Piperine and its derivative compounds were assayed via computational study for this inhibitory action. Different piperine derivatives with structural similarities were searched in pubchemcompounds[14], their structures were drawn in chemsketch[15], and their pdb structures were converted to energy minimized 3D structures using chimera software. The 3D structure of efflux protein Rv1258c was obtained from Itasser modeling online server which was then docked with all energy minimized 3D structures of piperine derivatives using iGEMDOCKv2.1[16]. In addition, comparative in silico study regarding anti-inflammatory activity of piperine and some anti-inflammatory drugs (NSAIDS) was done by docking with active site of COX1, COX2 enzymes responsible for prostaglandin synthesis.

Results and discussion

Identification of endophytic fungi: On the test plates, the vertical stem cuttings were placed such that the cut part is in contact with the solid agar media (Nutrient agar media) and incubated at room temperature for seven days. Whereas on control plate, surface sterilized but uncut stem parts were placed. Fungal growth was found only on the test plates (Figure-1B). It suggests the grown fungi to be an endophytes and at the same time efficacy of surface sterilization; i.e. removal of all possible microbes from stem surface (epiphytes). Long cylindrical shaped microscopic view of spores and fungal growth appearance as whitish mycelium with heavily intertwining hyphae were observed in the grown fungi. Under light microscope, the small cylindrical conidia spores of the fungi were clearly observed (Figure-1D). It provides the confirmation basis for the desired fungi i.e. Colletotrichum. These morphological observations were compared with figures given on Pictorial atlas of soil and seed fungi[17].

Table-1: Binding energies of Piperine derivatives with Rv1258c.

Ligand	Binding Energy (kcal/mol)
Rv1258c- piperitine	-104.914
Rv1258c-piperchabamidine	-93.2502
Rv1258c- piperonolanine	-93.1889
Rv1258c- chavicine	-92.1948
Rv1258c- piperine	-89.5951
Rv1258c-Piperanine	-83.4703

Table-2: Binding energies of Piperine and NSAIDs with the active site of enzyme COX2.

Ligand	Binding Energy (kcal/mol)
COX2-Piperine	-92.3838
COX2-Declofenac	-73.7821
COX2-Aspirin	-76.0094
COX2-Ibuprofin	-87.4612

Piperine extract and standard piperine have similar Rf (Retention factor) values in TLC analysis: The powdery form remained on RB flask, after extraction in rotary evaporator was dissolved in small volume of ethyl acetate to get crude extract which was then subjected for TLC analysis along with standard piperine as a positive control. Methanol: water (70:30) was used as the solvent system. The developed chromatogram stained with phosphomolybdic acid solution showed single color generation spot in case of standard piperine (Rf 0.78), while three such spots (with Rf values 0.94, 0.78, and 0.50) were observed in case of crude extract. In case of purified extract sample, single colored spot was observed with the Rf value 0.78. This strongly indicates presence of piperine alkaloid in crude extract along with some impurities and confirms the purified extract to contain piperine (Figure-2B).

Figure-2A: The non sporulated fungal hyphae was transferred from Martin's Rose Bengal Agar (MRBA) to Martin's Rose Bengal Broth (MRBB) and submerged fermentation of fungi was allowed to happen by incubation in shaking incubator at 30ºC for 30 days.

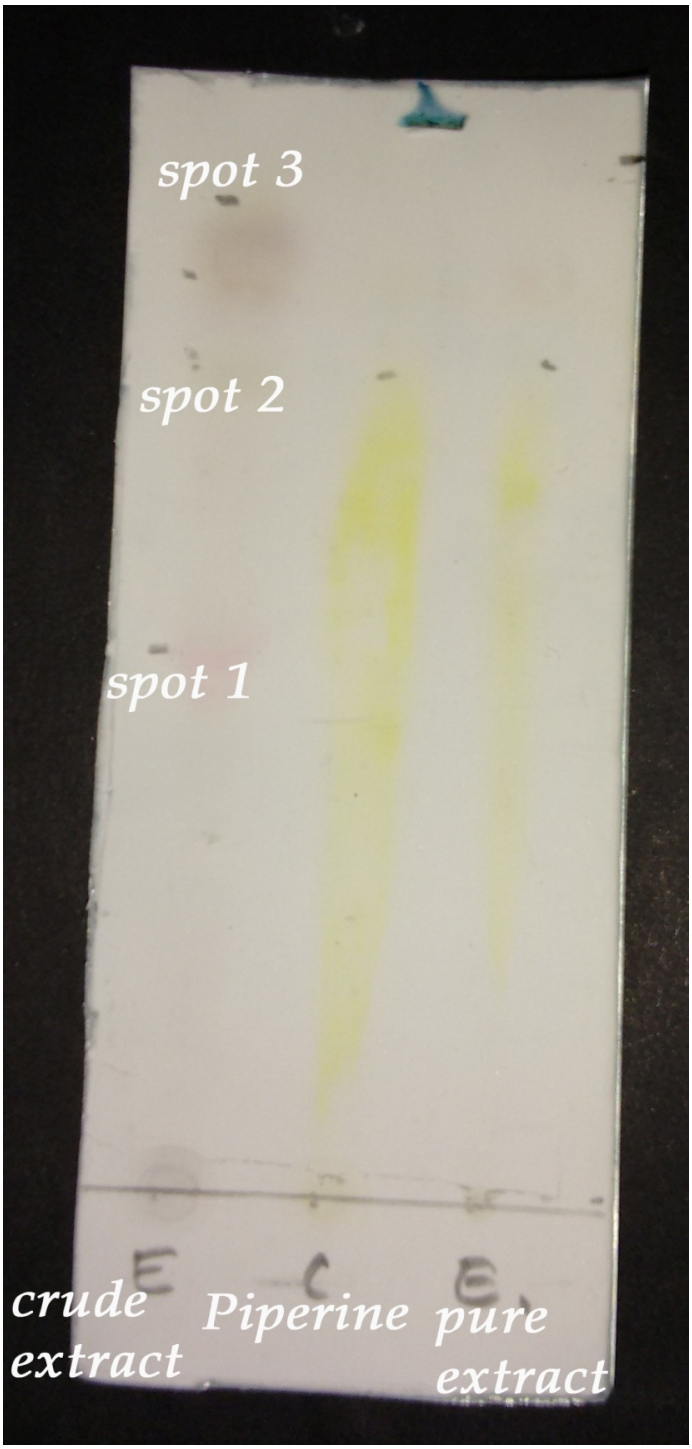


Figure-2B: Thin Layer Chromatography of crude extract (E), Standard Piperine (C), and pure piperine extract (E1); Methanol: water (70:30) was used as the solvent system.

Piperine extract have selective antimicrobial activity: Both piperine extract and standard piperine solution showed antibacterial activity against *Pseudomonas putida, Staphylococcus aureus* and*, Bacillus subtilis,* while no inhibition was found against *Escherichia coli* and *Aspergillus niger* (Figure-3). Maximum inhibition was observed against *Staphylococcus*. Slight inhibition was observed against *Saccharomyces cerevisiae* in case of both crude extract and standard piperine. No any inhibitory effect was shown by organic solvent (ethyl acetate) kept as negative control, showing no role of it in inhibition shown by crude extract and standard piperine.

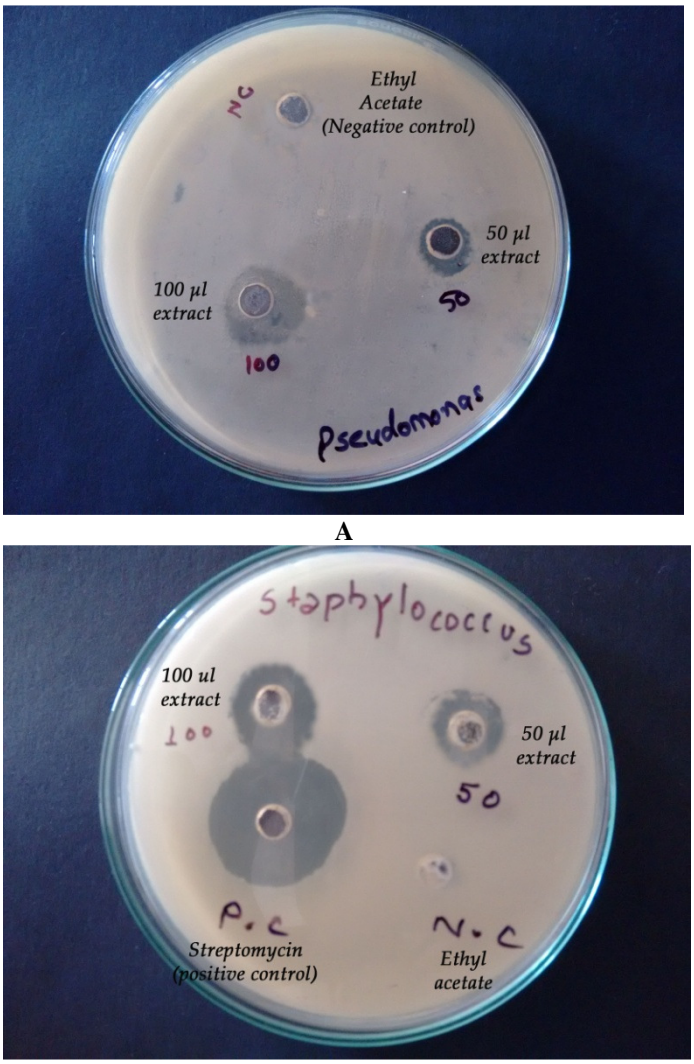
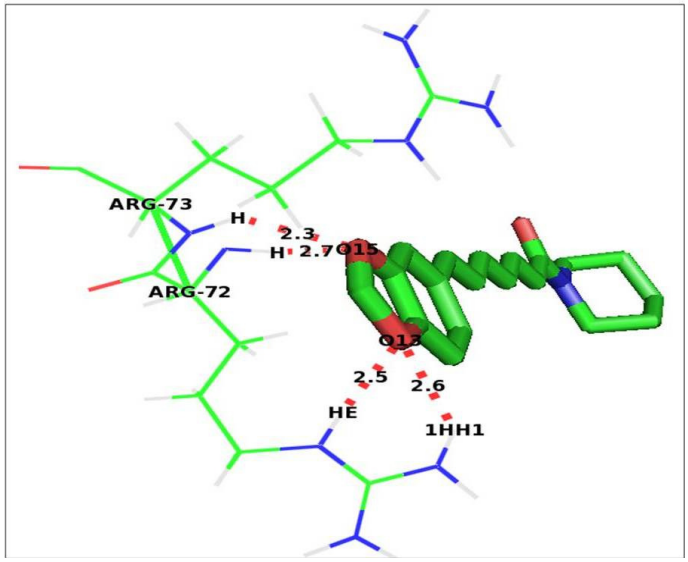


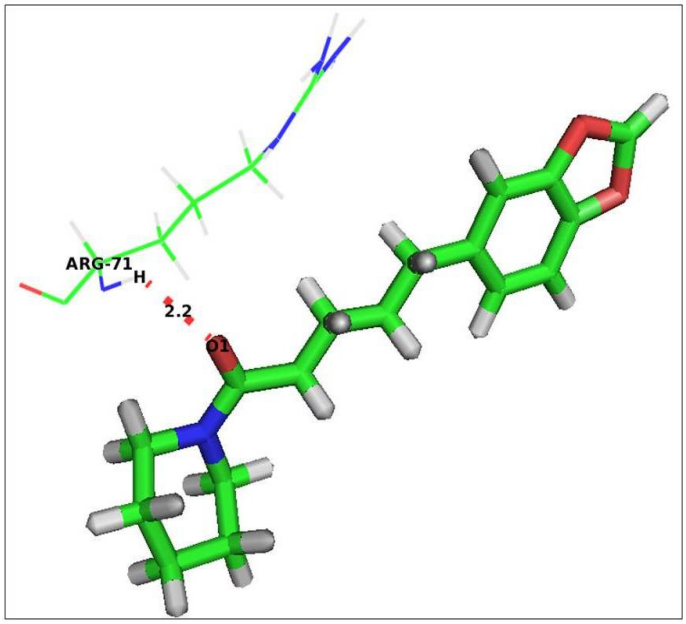
Figure-3: Antimicrobial study of Piperine extract.

***In silico* approach of piperine towards the antimycobacterial and anti- inflammatory activity:** The putative efflux protein of *Mycobacterium tuberculosis*; Rv1258c is responsible for resistance against anti tubercular drugs, where it effluxes them out of bacterial membrane. The autodocking of this protein with piperine and its derivatives (piperchabamide, piperonolanine, chavicine, piperitine and piperanine) showed piperitine to have least binding energy with active site of Rv1258c. Therefore, piperitine is likely to be most promising ligand amongst them for enhancing bioavailability of antitubercular drugs like rifampicin. Rv1258c making stable conformation compared to

Rv1258c-piperine having binding energy of -89.595 bonded - piperitine showed the free energy of binding to be -104.914 with four H- bonds, with active site by single H-bond (Figure-4). Piperine, on the other hand active site of prostaglandin synthesizing enzymes responsible of inflammation (-99.17 is likely to be potential anti-inflammatory ligand due to its least binding energy with COX1 and -92.38 with COX2). All the docked anti-inflammatory drugs (Aspirin, Declofenac, Ibuprofen) showed higher binding energy, suggesting the lesser inhibitory activity (Figure-5).

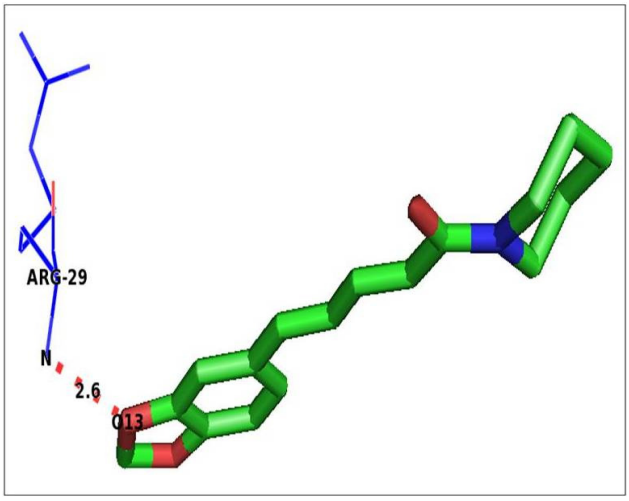


(A) Binding energies of several ligands; Piperitine - Rv1258c showed the least binding energy (-104.914).

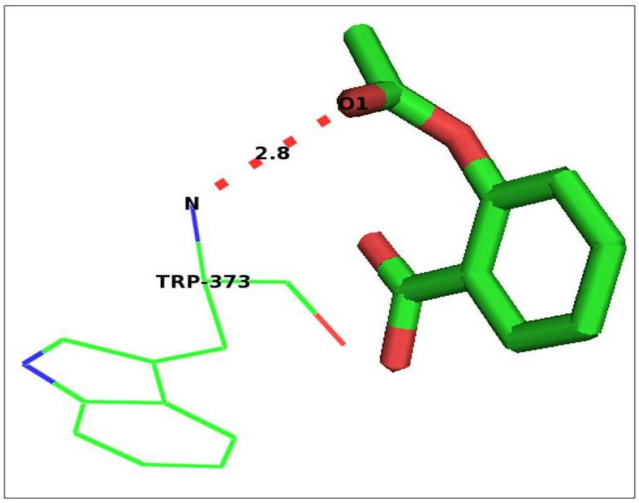


(B): Rv1258c-Piperitine autodocking result; Four H-bonds were observed in association with the active site of enzyme.

Figure-4: Autodocking of piperine and derivatives with active site of Rv1258c.



A: Binding energies of ligands (Piperine and NSAIDs) with the active site of enzyme COX2; Piperine showed the least binding energy of -92.3838 as compared to well known NSAIDs like Declofenac, Aspirin, and Ibuprofen



(B) Piperine – COX2 autodocking result.

Figure-5: Autodocking of piperine and some NSAIDs with the active site of Cyclooxygenases 2 (COX2).

Discussion: Various health related problems like drug resistance bacteria, parasitic protozoan, and fungal pathogens are alarming for the search of novel lead compounds of natural origin that can equally address to the problem of latent toxicity linked with chemically synthesized drugs. It is of common practice for centuries to use natural compounds, especially plant based, to address several health related problems. Almost half of the pharmaceutical drugs in clinical use today are based on natural products[18]. Plants not only produce plethora of bioactive compounds to cure illness, they provide unique shelter for wide varieties of endophytes also[19]. Majority of these endophytes are fungi and they are known to have biosynthetic capabilities even higher than host plant[20]. Plant endophytic fungi are believed to produce metabolites with less toxicity to the eukaryotic system which otherwise would result death of the plant host; especially

when concerned to normal fungi[21]. In order to maintain balanced antagonistic relation with host plant, endophytic fungi overcome the plant defense, only to the threshold level that allows them to inhabit. In addition, once tolerated, for the sake of stable association, endophyte starts synthesizing metabolites of host benefit that may be phytohormones or defensive compounds. These compounds have now been proved to have tremendous therapeutic potentials[22].

Genus piper is of high economic and medicinal value, consisting over 700 diversified species distributed all over the world. Plants of this genus are contributing principle ingredients in formulation of Indian Ayurvedic system to folklore medicine of Latin America and West Indies. This genus consists of more than one thousand species distributed especially in the equatorial regions. Their stem have noticeable sturdy node with strictly one simple leaf on each node[23]. Piper species, which are well known for pepper in worldwide spice market, have been widely researched for their phytochemical constituents and found to have several phytoactive compounds[24]. Some reported examples of piper species for therapeutic purposes are *Piper chaba* useful in asthma, bronchitis, fever, and abdominal pain; *P. aborescens* whose chloform extract shows significant activity against P-388 lymphocytic leukemia system in cell culture and *P. betle* extract showing anti-hypertensive activity. An endophyte named *Muscodor albus* MOW12 has been isolated from *Piper nigrum L.* that produces volatile antimicrobial compounds[25]. The pungent constituent of *Piper nigrum L.* (Black pepper); piperine is not only responsible for culinary uses; rather it is gaining significances for its medicinal and preservative properties. Studies based on modern cell, animal and human bodies have suggested piperine to have immunomodulatory, anti-inflammatory, anti-oxidant, anti-ulcer, anti-amoebic, anti-mycobacterial activities. Moreover, recent studies also proved it to have biotransformative effects including chemoprevention, detoxification, and enhancement of absorption and bioavailability of herbal and conventional drug[26]. Piperine has also been found to increase serum half lives of some nutritional substances like coenzyme Q10 and beta-carotene[27].

Our study as of now illustrated the successful culture of endophytic fungi *Colletotrichum gloeosporioides* from Piper nigrum L. and extraction of alkaloid piperine from submerged fungal fermentation. It has been shown that antimycobacterial drug Rifampicin, in combination with piperine results maximum efficiency even at lower concentration. This enhanced bioavailability is due to inhibition of putative efflux protein Rv1258c of *M. tuberculosis*[12]. Structurally, piperine consists of aromatic ring with a methylenedioxy bridge and piperidine ring forming an amide bond. These altogether are responsible for wide array of bioactivities. However, several modifications in these structural units give rise to its derivatives and these come up with altered biological properties (enhanced or sometime abolished)[28]. We performed computational study of autodocking piperine and its derivatives with active site of M. tuberculosis Rv1258c. We demonstrated the derivative piperitine as even more promising lead compound than piperine for increasing bioavailability of antimycobacterial drugs because of its better binding efficiency to this protein. Therefore, piperine comes up with possibilities of structural modifications that can result better therapeutic potentials like in piperitine. The pharmacological targets of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) like Aspirin and Declofenac are enzymes COX1 and COX2. These cyclooxygenases are responsible of biosynthesis of prostaglandins and these are targeted in therapeutics of pain and inflammation. COX1 is involved in homeostasis maintenance in most tissues, whereas, pro-inflammatory cytokines like TNFα, IL1, and IL6 enhances COX2 expressions in leukocytes. We performed comparative *in silico* study for the inhibition of COX2 by piperine and widely used NSAIDs viz. Aspirin, Declofenac, and Ibuprofen by autodocking of these ligand molecules with active site of COX2 enzyme. Results suggested more stable conformation of piperine-COX2 enzyme compared to widely used NSAIDs. Therefore, bioactivities of piperine involve the antibacterial as well as antifungal effects, which indicate their potential use in food industry and agricultural sectors.

Conclusion

We conclude that submerged fermentation of the endophyte *Colletotrichum gloeosporioides* and subsequent proper extraction can yield the pure extract of alkaloid piperine. Docking study indicates the potential application of piperine as antimycobacterial and antifungal compound. Since the production is via endophytic fungi and that too through submerged fermentation, it comes up with enormous scopes to take it to large - scale industrial production. For example-fermentation media optimization, nutritional characteristics study, and strain improvement. In addition, minor structural modifications in piperine leads to its derivatives, which can even retain better bioactivities.

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