
# ANTI BIOFILM ACTIVITY OF TRITERPENOIDS: AN OVERVIEW

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**Abstract:**

Phytochemicals, bioactive chemicals of plant origin, have long been used as herbal medicines in many countries but have become increasingly receptive in modern day biology for their non toxicity and easy availability. Among all the available groups, (like polyphenols, flavonoids, alkaloids) pentacyclictriterpenoids have received much attention for their wide range of therapeutic properties including anti-inflammatory, anti-cancer, anti-oxidant, anti-viral effects. They are further classified into three groups namely oleanane, ursane and lupane and Glycyrrhetinicacid (GA), ursolic acid (UA), and betulinic acid(BA) are three representative examples from each group respectively. All three compounds have been widely studied for establishing their anti-inflammatory, anti-cancer, anti-tumor and anti-microbial roles among many others. GA, UA and BA have also been studied for their anti-biofilm action. Anti-biofilm activity of various degrees have been established for all the triterpenoids against bacteria, particularly *Streptococcus mutans*, *Staphylococcus aureus* and other Gram positive pathogens and more recently against *Pseudomoans aeruginosa*. Though the precise mechanism of intervention remains elusive. Quorum sensing (QS) mediated alteration of gene expression has been associated with biofilm formation. In a systemic exploration for profiling action of petnacyclictriterpenoids against biofilms formed by Gram negative bacteria, GRA, UA and BA have been implicated in biofilm formation inhibition through impairing QS in *Vibrio choleare*, *Pseudomoans aeruginsoa* and *Acinetobacter baumannii* by interacting with various effectors of acylatedhomoserine lactone (AHL) based and autoinducer (AI) based QS-cascades. Within the scope of this review a brief retrospective of pentacyclictriterpenoid as candidate anti-biofilm agent is provided. Alongside, possible therapeutic applicability as a novel scaffold for drug development or in combination with antibiotics is also discussed.

**Key words:** Phytochemicals, Triterpenids, Quorum sensing, Biofilm, Pathogen

**Introduction:**

Phytochemicals, plant derived bioactive natural products, have been explored extensively for their therapeutic abilities since ages.Most prominent examples of such application includes curcumin from turmeric having anti tumor (Chang *et al.*,2020), anti microbial (Figueira *et al.*, 2020), anti inflammatory (Hasanzadeh *et al.*,2020) properties; gingerenone-A and shogaolfrom ginger with, grastroprotective (Haniadka *et al.*,2013) and anti obesity (Wang *et al.*, 2017) effects and catechols from tea with antioxidant and anti tumor potential (Tejero *et al.*,2007) to mention a few. Identification of structure function relationship of phytochemicals with biological macromolecules in combination with synthetic approach lead to tremendous augmentation of phytomedicine research.The biggest advantages of therapeutic application ofphytomedicines are its non toxic nature and apparent easy availability.With the receding life span of antibiotics due to the emergenceof resistance and the urge to find new anti-microbial agents have made the phytochemicals even more relevant for developing new-age antimicrobial interventions (Cowen *et al.*, 1999). Pentacyclictriterpenes, widespread in fruit peels, leaves, flower and stem bark have been identified as promising compounds with an array of pharmacological activities. This immensely potential group of molecules are broadly classified into three classes: the lupane-, oleanane-, and ursane, all harboring various pharmacologicaleffects.. Therefore, these triterpenes have been marked as promising lead compounds for the development of new multi-targeting bioactive agents (Jäger *et al.*, 2016). One of the major discoveries over the last few decades in the area of microbiology has been the realization that microbial growth and development takes place on a surface with the formation of a complex community like structure called Biofilm. Formation of biofilm structure allows the microorganism to withstand unfavorable environmental challenges, starvation, host immune system and other intervening agents like antibiotics, making them capable of causing a number of chronic disease conditions (Orazi *et al.*, 2019). Production of various pathogenic determinants as well as biofilm production is dependent on Quoramsensing (QS) (Yang *et al.*, 2020). Therefore disruption of QS has become a very useful alternate strategy to deal with the otherwise resistant pathogens. Any agents that can cause such disruptions are referred as quoramquenchers (QQ). Pentacyclictriterpenes, betulin and betulinic acid are being studied as candidate quorum quencher as these can perturb the QS response for biofilm formation by competitively inhibiting QS receptors in Gram negative pathogens like *Pseudomoans aeruginosa* (Rajkumari *et al.*, 2018). Antibiofilm action of petacyclictriterpenoids like oleanolic acid (OA), ursolic acid (UA) and maslinic acid (MA) has been expensively studied against Gram positive bacteria like *Staphylococcusaureus* and *Staphylococcus epidermidis* (Kurek *et al.*, 2014, Blanco-Cabra *et al.*, 2019)

Considering the immense potential of this group in albeit bacterial biofilms, within the scope of this review a comprehensive purview of research conducted to evaluate potential of pentacyclictriterpenoids as antibiofim agent so far is provided.

**Petacyclictriterpenoid a significant bioactive phytochemical:**

The number of bioactive phytochemicals synthesized by plants is enormous and most of them belong to phenols or their derivatives, flavonoid compounds, tannins, lignins and relatedcompounds. The main role of these being in plant defence against predatory attack of microbes, insects or others, these also provide odor (terpenoids), color (tannins, quinones)and flavor(terpenoids. Some are used by humans as spices (alkaloid in black pepper, terpenoids, tannins in cinnamon, terpenoidincloves, sulfated terpenoids in garlic, monosaccharide in coriander etc.) (Cowan, 1999) on a regular basis and impart immense health benefits. The gross categorization of phytochemicals with well documented antimicrobial action is summarized in Fig. 1.

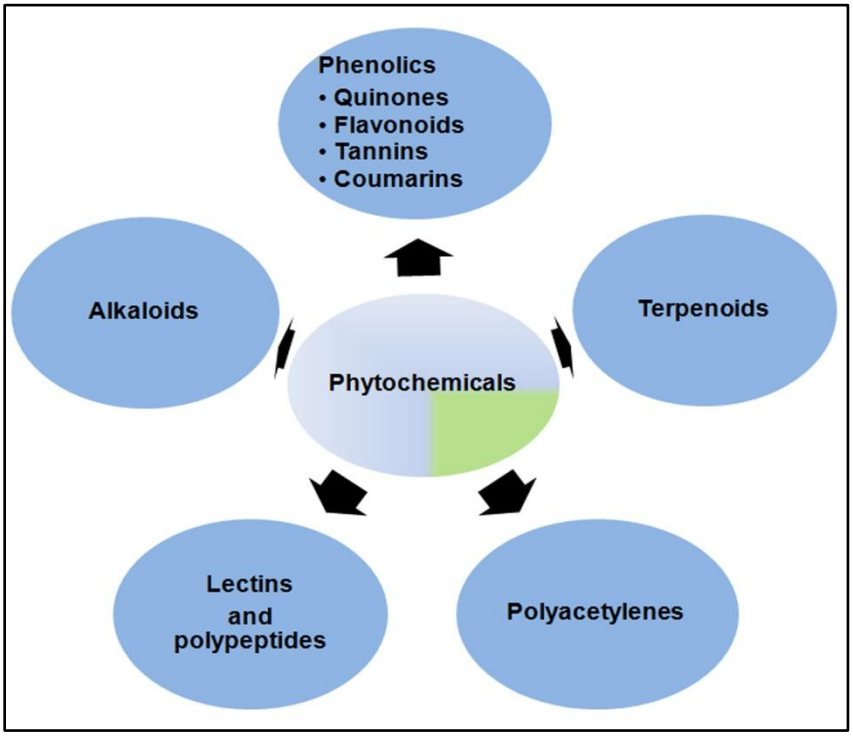


**Figure 1: Major groups of phytochemicals with medicinal importance**

Triterpenesare widely distributed in natureand are obtained either in free state, as esters or as glycosides. Triterpenoids are usually classified into three groups: acyclic, tetracyclic and pentacyclic (Alqahtani *et al.*, 2013). Pentacyclictriterpenoides are widely distributed in fruits (olive, sour cherry) fruit peels (tomato, apple, pear), leaves (rosemary, oregano, lavender), flower (clove, marigold) and bark (birch) (Sebastian Jäger et al, 2009).Pentacyclictriterpenoids are divided into three main classes, namely, oleanane, ursane and lupine, each of these classes comprising of bioactivecomponents (Jäger *et al.*, 2009). Each of these groups has received much attention due to their various biological pharmacological effects (Fig. 2, Table-1).

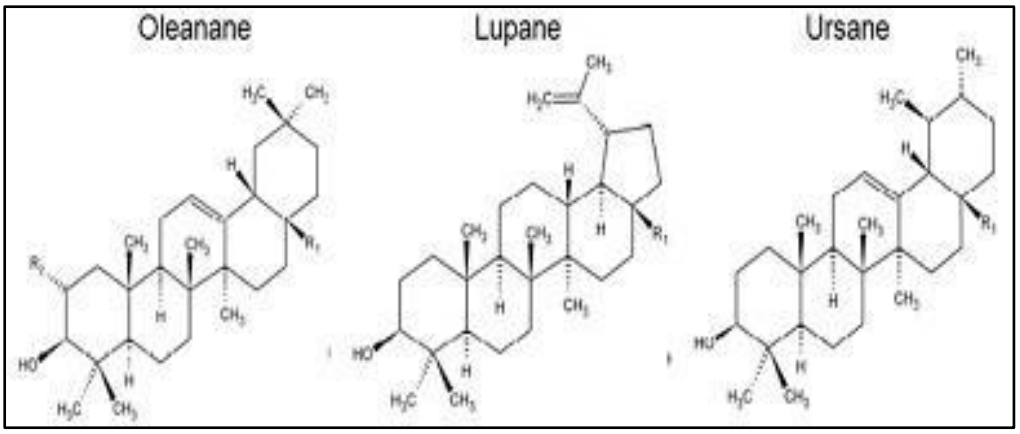


**Figure 2: Structural depiction of three major classes of pentacyclictriterpenoids. (Details of R1 and R2 are provided in Table 1)**

**Table 1: Classification and examples of pentacyclictriterpenoids (Jager *et al.*, 2009)**

| Triterpene family | Example | $R_1$ | $R_2$ |
|---|---|---|---|
| oleanane | □-amyrin | $CH_3$ | H |
| | oleanolic acid | COOH | H |
| lupane | lupeol | $CH_3$ | |
| | betulinic acid | COOH | |
| ursane | □-amyrin | $CH_3$ | |
| | ursolic acid | COOH | |

Glycyrrhetinic acid (GA) is a triterpenoid belonging to oleanane family. It is obtained largely from licorice, a leguminosaefound in the Mediterranean region, south Russia, centralAsia, northern China and America. Licorice has long been apart of thetraditional medicine

with its antibacterial, anti-viral, anti-inflammatory and calming effects (Oyama *et al.,* 2016). Extract of licorice contains significant amount of GA and have been studied extensively as well. GA and its various derivative have been assigned with many properties, including anti-inflammatory as well as antitumor (Markov *et al.,* 2018), anti-allergic (Yang *et al*., 2015), anti-filarial (Tyagi *et al.,* 2019) anti-viral (Perelmuter *et al.,* 1988) and anti bacterial (Yamashita *et al.,* 2019, Oyama *et al.,* 2016) activities. GA and its derivatives have also found to work as a pro-apoptotic agent (Logashenko *et al.,* 2011) proteasome activator, agent decelerating aging and Alzheimer's disease progression (Papaevgeniou *et al.,* 2016). Glycyrrhetinic acid in combination with other bioactive compounds acts against Dopamin receptor D3 for Parkinson's diseaseis (Mirza *et al.,* 2014). It has also been effective, alone or in association with other antibiotics against *Mycobacterium bovis* (Zhou *et al.,* 2012) and methicillin-resistant *Staphylococcus aureus* (de Breij *et al.,* 2016). Anti fungal (Kim *et al.,* 2013) and anti leismanial (Gupta *et al.,* 2015) activity has also been reported in past years. Ursolic acid (UA) is a pentacyclictriterpenoid belonging to the ursanefamily. UA is a secondary plant metabolite exhibiting a wide range of pharmaceutical properties. Ursolic acid, usually present in the stem bark (eucalyptus, black elder) leaves (oregano, rosemary, sage etc) or fruit peel (apple) (Woźniak *et al.,* 2015). Amongst various pharmacological properties of UA it's pulmonary, hepato, kidney and cerebro as well as osteoporosis (Woźniak *et al.,* 2015) are worth mentioning. Besides, ursolicacid also exhibits antioxidant and anti-inflammatory mechanisms (Habtemariam *et al.*,2019, Kashyap *et al.*,2016), anti cancer (Yin *et al.,* 2018, Chan *et al.,* 2019) anti viral (Tohmé *et al.,* 2019) anti-microbial activities against different strains of bacteria (Park *et al.,* 2018, Zhou *et al.,* 2017). This triterpenoid has also been exploited to manage neurodegenerative and psychiatric diseases (Ramos-Hryb *et al.,* 2017), obesity-induced cardiovascular diseases (Lin *et al.,* 2016), cardiomyopathy with diabetic condition (Woźniak *et al.,* 2015). A representative molecule from lupine family is betulinicacid (BA), which has been investigated highly in the past decade for an array of beneficial properties. BA and its analogues have been studied for anti-cancer (Lee *et al.,* 2019), anti-inflammatory Ekuadzi E *et al.,* 2018), anti-angiogenic (Shin *et al.,* 2011), immune-modulatory (Takada *et al.,* 2003), antimicrobial (Haque *et al.,* 2014), anti malarial (Innocente *et al.,* 2012), anti-tumor (Zhang X *et al.,* 2016), and anti-HIV (Li *et al.,* 2016) effects among many others. Besides therapeutic potential, BA has been explored againstchemically induced hypothyroidism (Afzal *et al.,* 2014). Although already established as a potent antimicrobial (Carvalho Junior *et al.,* 2019) agent, the anti-biofilm potential of these molecules are explored mostly in past one decade (Kannan *et al.,* 2019**,** Feuillolay *et al.,* 2016).

**Quorum sensing and biofilms:**

Biofilm is an assemblage of microbial cells surrounded by a matrix formed by the extracellular polymeric substances (EPS) secreted by those residing cells. A biofilm community can harbour a pure culture, but a community of mixed microbial species is more common in nature. Biofilm formation is a developmental process in which a quorum sensing signal molecule, an auto-inducer, functions to induce the secretion of the EPS and leads to the formation of characteristic three-dimensional biofilm architecture (Flemming *et al.,* 2016). Biofilm formation provide protection from toxic compounds, such as antibiotics, host immune response and predation (Sharma *et al.,* 2016), thus serve as a survival mechanism for the inhabitants. Almost all microorganisms can form biofilm and microorganisms most often (>99%) exist in nature as biofilms. Biofilm formation also protects microorganisms from various environmental challenges such as pH, salinity, and metal toxicity (Koo *et al.,* 2017) and even confers resistance to antibiotics and microbicides (Hall-Stoodley *et al.,* 2009).

Clinical research also revealed the importance of biofilm in infectious diseases. An estimated frequency of infections caused by biofilms, especially in the developed world, lies between 65% and 80% as per reports from Centres for Disease Control and Prevention (CDC) and National Institutes of Health (NIH), respectively (Moser *et al.,* 2018). Biofilm is significant for pathogenic bacteria as it modulates the pathogenic potential of bacteria as evident from cariogenic bacteria in plaque biofilms. Microorganisms in biofilms have been reported to be less susceptible to antimicrobial agents and have reduced sensitivity to inhibitors (Jabra-Rizk *et al.,* 2006). Biofilm formation results in delayed penetration of tobramycin and colistin into *Pseudomonas aeruginosa* cells (Musken *et al.,* 2018) and *Escherichia coli* biofilms exhibited decreased susceptibility to antibacterials (Schiebel *et al.,* 2017). Similar reports are available in ESKAPE pathogens (Pletzer *et al.,* 2018), in addition to high persistence rate after drug exposure to biofilms (Michiels *et al.,* 2016). Moreover, the potentially pathogenic bacteria like *Staphylococcus aureus, Enterococcus faecalis, Streptococcus, Klebsiella, Pseudomonas,* tend to grow on catheters, artificial joints, mechanical heart valves, etc which lead to persistent infections as a result of periodic release from the said focus (Costerton *et al.,* 2003). Biofilm dispersion is also a matter of clinical concern as release of cells from biofilms initiates a new round of infection. In *P. aeruginosa*, the localized depletion of nutrition in a biofilm has been hypothesized as inducer for release or detachment of cells from the biofilm (Chambers *et al.,* 2017).

QS is dependent on production of specific signalling molecules in population density dependent manner. These small molecules called autoinducers (AIs) are secreted by the cell and

once reaching upon a certain concentration, can bind to certain receptors present on the cell sending a designated signal leading to significant alteration in gene expression often related to the pathogenicity of the bacterium. Different virulence factors are regulated by QS for different bacterium like pyocyanin, lectin and other factors in *P. aeruginosa*, hemolysin and enterotoxin production in *S. aureus*, biofilm formation in *Vibrio*sp etc.

QS was first identified in *Vibrio* sp.. For regulating QS cascade the bacteria possesses two independent QS-inducers. The CAI-1 ((S)-3-hydroxytridecan-4-one) system that acts intragenously is uniqe for *Vibrio* (Kelly *et al.,* 2009). The Other auto inducer, AI-2 ((2S, 4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran borate), which is conserved across Gram negative bacteria for inter species communication (Chen *et al.,* 2002). *Vibrio* proteins CqsS and LuxPQ, functions as CAI-1 and AI-2 receptors respectively. At low cell density, and lesser level of autoinducers, CqsS and LuxPQ act as kinases for LuxU. The phosphate is funnelled to LuxO the key response regulator for the bacteria. When phosphorylated, LuxO triggers transcription of an array of four small RNA - Qrr 1-4. Cumulatively these small RNAs repress translation of HapR which is the master QS regulator in high cell density. Parallely, Qrr 1-4 RNAs activates translation of AphA, regulator for low cell density QS regulator. At high cell density, when auto inducer the CqsS and LuxPQ acts as phosphatse with successive dephosphorylation of LuxUand LuxO. Hence under such a situation, qrr 1-4 is not expressed. This leads to removal of repression and activation of HapR translation. On the flip side, translaton of AphR stalls.Under such situation the aggregative behaviour is induced. Jung *et al.* recently reported that two other QS receptor CqsR and VpsS, with unknown ligands integrate signal into QS cascade via LuxU (2015). Recently a third biofilm modulatory Qs system was discovered in *V. cholera*e. Autoinducer, called DPO (3, 5-dimethylpyrazin-2-ol), binds to a transcriptional regulator called VqmA. This complex activates expression ofaregulatory RNA VqmRwhicheventualy represses genes required for biofilm formation (Bridges *et al.,* 2019).

The bacterial QS signals mainly consistof acyl-homoserine lactones (AHLs), autoinducing peptides (AIPs), and autoinducer-2 (AI-2). The QS signal differ for Gram positive and Gram negative organisms. Gram positives rely on AIP signaling and Gram negatives on AHL signaling and both on AI-2signals as well. These three types of signaling molecules have been found to regulate growth and infectivity in bacteria. AHLs once accumulated upto the threshold level, diffuse across the cell membrane and bind target transcriptional regulators leading to gene expression(Yang *et al.,* 2009). For AIP, they upon reaching threshold, are transported out and then enter the cell by the help of a histidine kinase sensor which upon

phosphorylation alter expression of target genes. AI-2 system is used by the bacteria to receive signals from other species present in the same environment. For most of the bacteria, AI-2 signaling is carried out by Luxsynthetase. As biofilm formation has been intricately linked with QS for most of the pathogens along with expression of many other virulence factors, blockage of QS signaling is therefore considered as an efficient intervention strategy. Application of QS suppressors or Quoram Quenchers (QQ) to inhibit the expression of virulence factors and thus making them susceptible to host immune system seems like an efficient alternative therapeutic strategy. Inactivation of receptors, inhibition of synthesis of the signal, degradation of the signal and blockage of the signal using antibody are few of the strategies applied.

**Pentacyclictriterpenoid as quorum quencher:**

Over the years unrestricted use of antibiotics have made the issue of resistance more and more complicated. Hence finding suitable alternative has become an urgent need of the hour. Among many agents tried and tested, tritepenoids have been one of the most readily accepted one due to its non toxic nature. Many researchers have not only established these as potent anti microbial agents, but also anti biofilm agent against various groups of microorganisms.

In a study with oleanolic aldehyde coumarate (OALC), a triterpenoidcoumarateester and novel bioactive compound obtained from dalbergiatrichocarpa bark not only inhibited the formation of biofilm by *P.aeruginosa*, but also affected its maintenance. The compound found to interfere with the expression of the *las* and *rhl* mediated QS systems, Consequently QS-mediated virulence factors.AHL production was affected and external supply of AHL was unsuccessful to restore the condition proving the extent of damage even beyond AHL production (Rasamiravaka *et al.*, 2015). In a study by Rajkumari et al.betulin and betulinic acid were found to be strong competitive inhibitors of QS receptors, LasR and RhlR. Another two triterpenes, ursolic acid and resveratrol were tested for their anti biofilm potential against Methicillin Resistant *Staphylococcus aureus*. Although ursolic acid seemed to inhibit biofilm formation by affecting amino acid metabolism, resveratrol affected QS related gene expression. *Hld gene that codes δ-hemolysin and located within the agr locus, one of the QS clusters in S.aureus,* was found to be up-regulated, indicating that the role of resveratrol and ursolic acid in MRSA *agr* function at the RNA level for inhibiting biofilm formation. A similar study by Quave CL et al with oleanene and ursene derivatives from European Chestnut leaf extracts showed biofilm inhibition in *Staphylococcus aureus* by targeting agr alleles (Quave *et al.*, 2015). Five limonoids isolated from sour orange were checked for their ability to interfere with QS and biofilm formation in *Vibrio harveyi*. Out of the five tested four, namely isolimonic acid, deacetylnomilinic acid glucoside

and ichangin were found to inhibit AI mediated QS. Moreover isolimonic acid and ichangin, both were identified as potent modulator of luxO expression (Vikram *et al.*, 2011). In a comprehensive study with three triterpenoids, glycyrrhetinic acid, ursolic acid and betulinic acid against *Vibrio cholerae* biofilm, all three were found to interfere with QS process and perturb biofilm formation (Fig.3). Molecular docking analysis hinted about probable interaction with cyclic di-GMP sensor VpsT, autoinducer-2 sensor kinase LuxP-LuxQ and transcriptional activator HapR (doi: https://doi.org/10.1101/2020.01.06.896183). Over all, comprehensive and better understanding of anti-biofilm potential of various pentacyclictriterpenoids has offered the scope of developing multi-faceted strategies to combat bacterial infections.

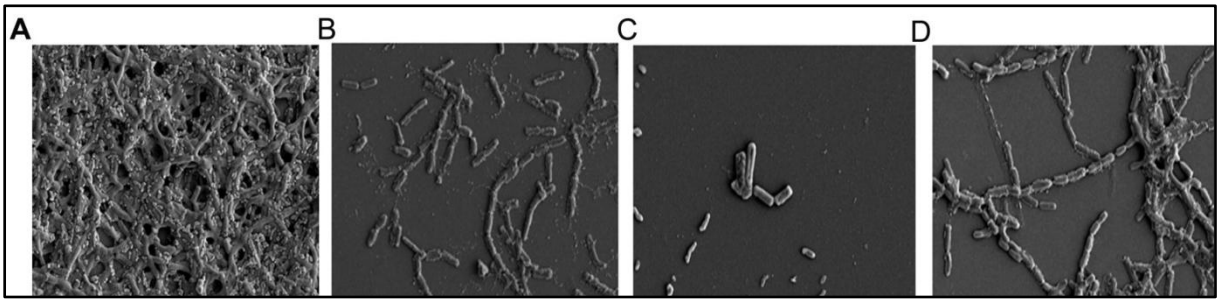


**Figure 3: Effect if triterpenoids on biofilm integrity. Log phase *Vibrio cholerae* cells were allowed to form static biofilm in absence (A) or presence of three different pentacyclictriterpemoids representing oleanone(B), lupine (C) and ursane (D) family. Integrity of biofiulmswere visualized by scanning electron microscopy (Data: Paul Bhattacharya *et al.*, Unpublished).**

**Concluding remark:**

A major concern for clinical implication of the pentacyclicterpepenoids is cytotoxic impact on various mammalian cell lines. However, the triterpenoids can offeran excellent bioactive scaffold to impart biofilm selectivity and diminishing cytotoxicity. Selective nano-delivery strategy for biofilm can also accentuate anti-biofilm action. Testing in combination with antibiotic for optimal triterpenoid-antibiotic composition can culminate into successful combinatorial therapeutics.

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