

1 **Cellular internalization of Godanti Bhasma (anhydrous CaSO₄) induces**
2 **massive cytoplasmic reversible vacuolation and survival response of**
3 **mammalian cells.**

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20

21 **Abstract**

22 Cellular internalization and intracellular trafficking of particles depend on their specific
23 physicochemical properties. Godanti Bhasma (GB) is a traditional Indian medicine formulation
24 prepared by heating of gypsum powder with herbal extracts. Chemically, GB is anhydrous
25 calcium sulfate. During formulation it obtains unique physicochemical properties that lead to
26 its rapid cellular internalization and induction of massive cytoplasmic vacuolation. Interestingly,
27 no cellular internalization was found with parent gypsum particle. Flow cytometry analysis and
28 live tracking of GB treated cell showed particle internalization, vacuole formation, particle
29 dissolution and later vacuolar turnover. GB particle dissolution in acidic cell free solution
30 mimics intravacuolar environment where GB was disintegrated under acidic pH suggesting
31 lysosomal enzymes might have no role in GB degradation. Vacuolation often accompany with
32 the sign of cell death whereas, in our study, massive vacuolation by GB did not induce any cell
33 death. Moreover, GB treated cells survive with complete vacuolar process, which was reversed
34 following post-treatment with vacuole inhibitors in GB treated cells, suggesting normal vacuolar
35 function is essential for cell survival. In immunoblotting, upregulation of LC3-II was found in
36 GB treated cells. Treatment of the cells with GB was also found to induce translocation of the
37 LC3 protein from the nucleus to vacuolar membrane by immune-cytochemistry, indicating LC3
38 associated phagocytosis (LAP) like function. This was found to be reversed in the cells treated
39 with vacuole inhibitors. The vacuolar function essential for cell survival, preserves mechanistic
40 correlation with LC3 lipidation on vacuolar membrane, intracellular controlled and slow
41 degradation of GB particle and further vacuolar turnover limiting swelling pressure in cells.

42

43 **Keywords:** Gypsum, Anhydrous calcium sulfate, Phagocytosis, Vacuole biogenesis, Lysosomal
44 activity, Particle degradation.

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47

48 **Introduction**

49 Eukaryotic cells develop intracellular membrane-bound organelles that compartmentalize
50 biochemical and biophysical processes essential for cellular functions. Cytoplasmic vacuoles are
51 one member of the organelles that serves a variety of functions including; secretory, excretory,
52 and storage. Unlike plant and fungi cells, most animal cells commonly do not contain vacuoles
53 as regular organelles. The vacuoles in animal cells are very smaller than plant and fungi,
54 enriched with hydrolytic enzymes called lysosome. These are about 0.2-2 μm in diameter and
55 acidic in nature. Irreversible cytoplasmic vacuolization has been found in animal cells, when cell
56 is infected by some viral and bacterial pathogens [1-4] as well as with the treatment of various
57 natural and artificial low-molecular-weight compounds including medicinal drugs and industrial
58 pollutants [5-11]. In most of the cases, irreversible cytoplasmic vacuolation is related to cell
59 toxicity. In contrast to irreversible vacuolation, reversible vacuolation occurs as long as chemical
60 compounds are present in medium suggesting vacuolization is a reversible process. But
61 prolonged exposure of the chemical causes vacuole irreversible and eventually cell death [12-
62 14].

63 Vacuolation is initiated by the interaction of cell membrane and particles. Researchers have tried
64 to understand the response of the biological system during and after particle uptake in different
65 cells and tissue model [15-17]. The mechanism of particle uptake occurs either by diffusion or
66 endocytosis. Endocytosis is a process where extracellular or cell surface particle is internalized
67 by the invagination as well as pinching off the plasma membrane. Usually, larger particles (>0.5
68 μm) internalized by phagocytosis that occurs in restricted cells (macrophage, monocyte) [18].
69 However, internalization of the larger particle by non-professional-phagocytic cells is also
70 reported [19-21]. Phagocytosis is triggered by the interaction of the particle-bound ligand with
71 receptors on the surface of the cell membrane; then the particle is delivered into a sealed
72 intracellular vacuole i.e. phagosome [22-24]. The nascent phagosome matures by interaction
73 either with endosomal vacuoles or lysosomes or both to develop phagolysosome hybrids
74 enabling the transfer of lysosomal hydrolytic enzymes that mediate degradation of vacuolar
75 cargo [25-27]. V-ATPase delivered from the membranes of the endocytic pathway during
76 phagolysosome formation is responsible for the maintenance of acidification in the vacuolar
77 lumen [28, 29]. The maintenance of acidification in the vacuolar lumen is crucial for the
78 hydrolytic activity of the lysosomal enzymes. Various unwanted biomolecules including

79 proteins, nucleic acids, carbohydrates, and lipids are broken down by the hydrolytic enzymes in
80 an acidic condition in the vacuolar lumen. The strategy of vacuole biogenesis in response to
81 extracellular particle, and its further structural and functional maintenance is very important for
82 the survival of cells. When the vacuolar function is impaired, cells become stressed and
83 eventually cell death occurs. However, a precise mammalian cell-based model system for
84 phagocytic pathway has not yet been discovered.

85 For the first time, vacuole biogenesis in mammalian cell (non-phagocytic) was observed by one
86 of the traditional Indian drug named Godanti Bhasma (GB). Bhasmas are unique Ayurvedic
87 medicines, prepared by incineration of metals or minerals particles processed with the herbal
88 extract [30]. In India, therapeutic uses of these medicines are commonly evidenced since the 7th
89 century [31]. GB is a traditional ayurvedic medicine for digestive impairment, headache, chronic
90 fever, cough, dyspnoea, phthisis leucorrhoea and emaciation in children [32, 33]. It is also used
91 in migraine [34], osteoarthritis [35] and gastric ulcer [36]. However, the mechanism of action of
92 the drug has yet not been studied.

93 In the present study, we assessed *in-vitro* cell-based assay to know how cells interact with GB
94 particle and following mechanism of vacuole biogenesis and internalized particle degradation.

95

96 **Results**

97 **Godanti Bhasma; Anhydrous phase of gypsum**

98 In Ayurveda, incineration procedure plays an important role in transformation of metals or
99 minerals into therapeutic form. In the present study, GB is prepared through incineration of
100 gypsum powder at 800 °C temperature. Here we characterized the structural difference of
101 gypsum and GB particle by using Confocal Raman in the range of 20-4000 cm⁻¹, and FT-IR in
102 the range of 600-4000 cm⁻¹. Both vibrational spectroscopy techniques are versatile in probing
103 structural changes and dehydration in minerals. In Raman spectroscopy (Fig. 1A), the presence
104 of water in gypsum was detected by its two characteristic absorption bands at 3494 and 3406 cm⁻¹
105 ¹ regions showing O-H stretching mode of gypsum which was disappeared in the GB (anhydrite
106 phase). The formation of phase wise anhydrite forms of CaSO₄-H₂O system was confirmed by
107 the complete disappearance of water molecules while heated at 110 to 1300°C [37]. Further, the
108 main Raman band centered at 1008 cm⁻¹ in gypsum was shifted to 1017 cm⁻¹ in GB. These are
109 ν_1 symmetric stretch vibration modes of SO₄, up-shifted following the alteration of the hydration

110 degree. Both compounds exhibited doublet for ν_2 symmetric bending of SO_4 at 415, 494 cm^{-1}
111 and 416, 497 cm^{-1} in gypsum and GB, respectively. The peaks at 1136 cm^{-1} in gypsum were
112 bifurcated at 1127, 1158 cm^{-1} in GB revealed SO_4 in ν_3 asymmetric stretch vibration modes.
113 The peaks observed at 619 and 671 cm^{-1} in gypsum and 608, 625 and 674 cm^{-1} in anhydrite
114 gypsum are ν_4 (SO_4) asymmetric bending vibration modes. All these observations strongly
115 indicate structural changes involving the rearrangement of sulfate ions and O-H stretching during
116 the transition of gypsum to GB with an onset temperature at 800 °C. The details of Raman
117 spectra of gypsum and GB are summarized in Table S1 comparing the observed internal modes
118 made between $\text{CaSO}_4\text{-H}_2\text{O}$ system (hydrous to anhydrite phases). Some Raman spectra observed
119 at 129, 177 cm^{-1} in gypsum and 126, 168 cm^{-1} in GB were attributed to Ca ion. The thermal
120 transformation of gypsum was studied extensively by many authors [38-40].

121 The FT-IR spectrum of gypsum centered at 3527 and 3387 cm^{-1} was assigned to O-H bending
122 vibrations, and the band at 1679 and 1617 cm^{-1} corresponds to O-H anti-symmetric stretching
123 indicates crystalline water molecules in gypsum which were disappeared in anhydrous GB. The
124 band at 1091 cm^{-1} in gypsum and 1097 cm^{-1} in GB associated with the stretching vibrations
125 mode of the sulfate (SO_4)⁴⁰ (Fig. S1).

126 Surface morphology and elemental composition of GB particle were analyzed by FESEM and
127 EDX, respectively. The SEM analysis demonstrated the irregular shape with different size of GB
128 particles ranging mean size of 0.5–5 μm (Fig. 1B and 1D). The EDX was used to detect elemental
129 composition of Bhasma particle as calcium sulfate (Fig. 1C).

130 Zeta potentials were measured to evaluate electrochemical changes at the microparticle surface due to
131 the thermal transformation from gypsum to GB. Gypsum microparticles showed a negative Z
132 potential -10.42 ± 0.98 and -11.1 ± 0.45 , whereas GB particles altered the surface charge towards
133 neutral -1.33 ± 2.65 and -1.75 ± 2.41 in 10% FBS and water suspension, respectively. However,
134 near to neutral zeta potential of particles tend to aggregate faster due to the less physical stability
135 of the colloidal systems [41].

136

137 **Godanti bhasma induced massive vacuolation in cells**

138 In the present study, we observed massive vacuolation in the cytoplasm with various sizes of the
139 vacuole in 3T3-L1 cells exposed to the GB (Fig. 2B & S3), whereas raw gypsum powder was
140 unable to induce any vacuolation (Fig. 2C). The vacuoles were stained with neutral red

141 indicating their acidic nature (Fig. 2D). As pH in lysosome and vacuoles are lower than the
142 cytoplasm, the dye penetrates inside vacuole, becomes charged and retained inside the lysosomes
143 and vacuoles. Quantification of GB induced vacuolation was done by neutral red uptake assay in
144 cells. A dose-dependent response was observed in 3T3-L1 cells exposed to GB with various
145 concentrations, the vacuolation increased gradually according to the gradual increasing
146 concentration of bhasma particles (Fig. 2G). In time course experiment, vacuolation was
147 increased up to 24 h of bhasma treatment (Fig. 2H). It is also noted that nascent vacuoles first
148 appeared around the perinuclear region within 2-3 hours of GB treatment, and a gradual increase
149 in the number and size of vacuoles was observed until much of the cytoplasm occupied by a
150 single or several vacuoles (Fig. S2). In our experiment, the bhasma particles were precipitated
151 due to the gravitational force on the surface of the cell. However, the vacuoles were formed as
152 long as particles are available in the culture medium. The size of vacuole approximately 1–70
153 μm in diameter was observed after 24 h of bhasma treatment (Fig. S3). To the best of our
154 knowledge, extraordinarily large vacuoles were shown to induce in mammalian cells.

155 It was shown that GB induced vacuole formation was inhibited by Bafilomycin A1 (BFA1)
156 indicating vacuolation require V-ATPase enzymes (Fig. 2E) that supply H^+ to vacuole from
157 cytoplasm. Further we found that Chloroquine (CQ) was also inhibited GB induced vacuolation
158 (Fig. 2F). CQ is a lysosomotropic weak base; it diffuses into lysosome and vacuole where it
159 becomes protonated and trapped. The protonated CQ then increases the vacuolar pH. The kinetic
160 study of GB induced vacuolation was also done to identify the minimal concentration of two
161 lysosomal-vacuolar inhibitors (BFA1 and CQ) required to block the vacuolation process in 3T3-
162 L1 (Fig. 2I and 2J). The vacuolation was suppressed up to 120 nM of BFA1 whereas, vacuolar
163 size and number was found to increase gradually up to 2 nM concentration. In all the above
164 tested concentrations, cells showed 100% viability. In the case of CQ treatment, GB induced
165 vacuolation was suppressed up to 2 μM concentration, gradual vacuole size was shown to
166 increase in lower concentrations up to 0.5 μM .

167 To see the specificity and degree of the vacuolation, we examined total of seven cell lines 3T3-
168 L1, Neuro 2a, A549, MDA-MB231, MCF-7, HCT-116, and HeLa. Interestingly, all the non-
169 phagocytic cells exhibited a similar pattern of vacuolation after 24 h of GB treatment (Fig. S 4).

170

171

172 **Cellular internalization of GB particle**

173 Initially FESEM was used to identify the vacuole and particle inside the vacuole within cells.
174 FESEM analysis clearly showed the presence of complete vacuole and particle inside of it (Fig.
175 S5 A&B), but EDX did not show any spectral change as EDX was not able to detect in intact
176 cells. To know the elemental analysis of particle inside the vacuole, we performed transverse
177 sections (10 μm) across the fixed cells to expose the particles, and then further cell was
178 analyzed through FESEM and EDX. The results clearly indicated that vacuole contains particle
179 composing calcium, sulfur and oxygen elements which were confirmed through FESEM as well
180 as EDX spectra of sectioned cells. These elements correspond to the GB particle inside the
181 vacuole (Fig. S5 C&D).

182 We investigated the internalization of GB particle in cells by flow cytometer analysis (Fig. 3).
183 Cells were incubated with bhasma particles at different time points starting from 30 min to 12 h.
184 The pictures showed that particles were localized on the surface of cells at 30 min of treatment
185 (Fig. 3B). Membrane invagination was found at the site of particle attachment on the surface of
186 the membrane and after its attachment a circular extension or curvature of the membrane was
187 observed around the surface of the attachment which might be the phagocytic cup formation
188 (Fig. 3C). The phagocytic cup was studied extensively by many authors [23, 42, 43]. After
189 internalization, particles were associated with membrane-enclosed vacuoles presumably
190 trafficking to the endocytic vacuolar system. It was clearly found that the size of vacuoles
191 increased over time (Fig. 3D).

192

193 **GB Particle degradation inside the vacuole**

194 To know the GB particle degradation, flow cytometry analysis of isolated particles from cell
195 lysate was done at different time points (1 to 24 h) of GB treatment in 3T3-L1 cells (Fig. 4A).
196 The result revealed that most of the particles were associated with cells at 1 h of GB treatment.
197 The particles at this stage were fully internalized or going to be internalized. However, 1 h
198 culture media contains a certain amount of particles which were not internalized. The particle
199 internalization in cells is a continuous process according to the availability of particles in the
200 culture media. In the same figure, it is clearly shown that the particles were internalized and
201 further degraded over times up to 24 h. Particles were completely degraded by cells and no

202 particles were available in culture media after 24 h. It seems that the sample with GB treatment
203 at 24 h was same as samples of untreated control cells.

204 The particle degradation inside the vacuole was also confirmed by time-lapse microscopy
205 targeting a single cell (Fig. 4B). It was clearly found that the particle was degraded into smaller
206 particle over time and finally disappeared from the vacuole. In supporting information Movie-1
207 we found that most of the particles were rapidly internalized by 3T3-L1 over the first 1-3 h of
208 incubation. Co-treatment of GB with either BFA1 or CQ inhibits vacuolation process. It was
209 clearly found that in the presence of inhibitor, GB particles were attached on the surface of the
210 membrane but not internalized as further acidification was stopped. Therefore following
211 internalization of the particle, acidification is required to make vacuole. After the disappearance
212 of particle the vacuoles were also decreased in size over time and finally disappear from
213 cytoplasm restoring the normal morphology of cells (Fig. 4B and Movie 1)

214

215 **Bhasma particles degradation is associated with lysosomal activity inside the vacuole**

216 Lysosomes contain many hydrolytic enzymes such as nucleases, proteases, and lipases. The
217 hydrolytic enzymes require an acidic environment for their optimal activity, and lysosome
218 provides this by maintaining low pH in its interior. In eukaryotes, lysosomes allow vacuolar
219 digestion by fusing with endocytic vacuoles (endosomes and phagosomes) [25-27]. The H⁺
220 pump (V-ATPase) in the lysosomal membrane, as well as endocytic vacuoles, uses the ATP to
221 pump H⁺ into the vacuolar lumen, thereby maintaining the lumen at its acidic pH [28,29].
222 Acridine orange (AO) is a pH indicator in live cells, it emits red fluorescence at acidic pH and
223 green fluorescence at physiological pH. We used acridine orange to monitor intracellular pH
224 in vacuoles in cultured cells at different time points of GB treatment (Fig. 5A). At 1 h of
225 particle addition, AO was detected mainly in the perinuclear region as small lysosomal
226 compartments. At 3 h of treatment, AO stained in round shaped granular structure in the
227 perinuclear region of cells. This indicated that the granular structure is the endocytic vacuoles
228 which are acidic in nature. At 12 and 24 h, the vacuoles were huge and also emit red
229 fluorescence. The GB particles inside the vacuoles might have a possibility of degradation
230 directly in the vacuolar acidic environment. However lysosomal degradation is done by
231 lysosomal hydrolytic enzymes in acidic condition.

232

233 **Mimicking vacuolar pH environment in cell-free environment**

234 The dynamic process of vacuole biogenesis involves particle internalization, phagosome
235 formation, fusion of endocytic pathway, and finally phagolysosome formation. The pH of these
236 vacuolar organelles ranges from 6.5-5.0, while pH of the primary lysosome is 4.5 [20, 44, 45].
237 We examined the solubility of GB under physiological conditions (pH 7.4) to
238 vacuolar/lysosomal acidic conditions (pH 4.5) in cell free environment. GB particles were
239 incubated in different pH condition (4.0-7.5) of PBS buffer solution, and turbidity test (580 nm)
240 was done in time points 15 min, 90 min, 3, 6, 12 and 24 h (Fig. 5B). In the buffer solution of pH
241 7.5, GB maintained their original size indicating that GB was insoluble at physiological
242 conditions. By contrast, GB was disintegrated under acidic conditions at a pH value similar to
243 phagolysosomes. This size of GB particle at physiological conditions was larger where these
244 particles were dissolved under acidic conditions (Fig. 5C), which is consistent with the
245 assumption that particle was degraded to a smaller size in vacuole because anhydrous calcium
246 sulfate shows solubility at pH range 4-6.

247

248 **Godanti Bhasma induced vacuolation does not affect cell viability and proliferation**

249 Initially, 3T3-L1 cells were treated with different concentrations (0-2.5 mg/ml) of GB for a
250 period of 24 h to test its effect on cell viability. After endpoints, no significant signs of toxicity
251 were observed in all tested concentrations of GB in 3T3-L1 cells (values over 100% should be
252 considered as complete viability). Further, the effect was examined after the 48, 72 and 96 h of
253 treatment on the cell viability of 3T3-L1 (Fig. 6A) and it was not found toxic to the cells. To
254 explore the cytotoxic effects of GB at various concentrations (0-2.5 mg/ml) in six other cells lines
255 (Neuro 2a, A549, MDA-MB231, MCF-7, HCT-116, and HeLa), cells were analyzed after 24 h of
256 treatment; no toxicity was observed (Fig. 6B). Furthermore, the effects of GB on cell proliferation
257 were investigated using *in vitro* scratch assay. The cell-free scratch area in control, as well as GB,
258 treated 3T3-L1 cells were closed at 8 h post-scratch (Fig. 6C). This finding clearly indicated that
259 GB does not cause any significant effect on cell viability as well as cell proliferation.

260 Activation of the caspase-3 pathway is a characteristic of apoptosis, and to determine the
261 involvement of caspase-3 in GB induced vacuolation, the activation of caspase-3 by colorimetric
262 caspase-3 assay system at different time points was examined. Exposure of 3T3-L1 cells to
263 Bhasma particles (0.15 mg/ml) for 0, 2.5, 5, 10 and 24 h caused no increase in caspase-3 activity

264 compared to a positive control (caspase-3). Untreated cells were used as a negative control (data
265 not shown).

266

267 **Post treatment of vacuole inhibitors BFA1 and CQ promote cytotoxicity in GB induced** 268 **vacuolated cells**

269 In our experiment, we found that GB induces massive vacuolation without any cell death. The
270 vacuoles were reversible as particles were degraded and following vacuolar turnover. To prove
271 the vacuolar function in survival of GB induced vacuolated cells, we introduced vacuole
272 inhibitor BFA1 (100nM) and CQ (1 μ M) after 24 hours of GB treatment in 3T3-L1 cells. We
273 performed cell toxicity assay after 48 hours of the inhibitors addition, and found a significant cell
274 death in GB+BFA1 (Fig 6D) and GB+CQ (Fig 6E) compared to respective controls. The result
275 indicated that cell death occurs due to irreversible or defective vacuole by inhibition of vacuolar
276 function with the post treatment of the vacuole inhibitors.

277

278 **GB induced vacuolation is associated with LAP like function**

279 LC3-associated phagocytosis (LAP) is a phenomenon distinct from autophagy, wherein LC3
280 translocation occurs to particle containing phagosome. To identify the LAP-like LC3 lipidation,
281 we performed immunocytochemistry with LC3A/B antibody in GB induced vacuolated cells
282 (Fig. 7A and S6). The result indicated that LC3 protein is accumulated in the nucleus of
283 untreated cells, whereas in GB treatment it translocated to the membrane of vacuoles, suggesting
284 activation of LAP like function in GB induced vacuolated cells. However, cytoplasm and
285 nucleus also stained with LC3 antibody. To understand the relation between LAP like function
286 and vacuole inhibitor BFA1 in GB treated cells, post treatment of BFA1 (100nM) was done in
287 GB induced vacuolated cells for 18 h. We found that the accumulation of LC3 (lipidated LC3)
288 was suppressed on the vacuolar membrane/periphery, whereas LC3 expressed in whole cell. The
289 present findings indicated that the LC3 protein have an important role in GB induced vacuolar
290 function through LAP like mechanism. Also, we estimated LC3 expression in cell lysate of
291 3T3L1 in different time points after GB addition compared to untreated control and CQ
292 treatment (autophagy inhibitor) (Fig. 7B). The expression of LC3 increased in GB treated
293 samples compared to untreated control where as in CQ it was highly expressed. The result
294 indicated that the expression of LC3 is in steady state in GB treatment suggesting LAP like

295 function involved in vacuolar process. However, in CQ treatment autophagosome
296 accumulation is enhanced due to inhibition of Autophagy flux. To understand the role of
297 vacuole inhibitors in LAP mechanism, we examined LC3 expression with GB, BFA1 and CQ
298 alone and GB combined with post BFA1 or CQ treatment in 3T3L1 cells by western blotting
299 (Fig. 7C). The cell lysates were prepared after 18 h of BFA1/CQ treatment. We found that LC3
300 was highly expressed in presence of BFA1 and CQ with or without GB treatment. Whereas
301 moderate LC3 expression was found in only GB treatment compared to untreated control. We
302 also performed the same experiment in Neuro 2a cells (Fig. 7D), and found that the same
303 expression pattern as of 3T3L1 cells. The result indicated that LAP like mechanism is
304 functional in vacuolated cells wherein it is abolished with the post treatment of vacuole
305 inhibitors.

306

307 **Discussion**

308 The present study sheds lights on the vacuole biogenesis induced by Godanti bhasma (GB) and
309 downstream vacuolar progression in animal cells. In this study, the most interesting phenomenon
310 is that massive vacuolation was observed in mammalian (non-phagocytic) cells with the
311 treatment of GB (an anhydrous CaSO_4), whereas parent gypsum (dihydrate CaSO_4) did not
312 induce any vacuolation. To know this phenomenon, initially we analyzed the structural changes
313 in gypsum and GB. The change of crystal structure on heating of gypsum was clearly evaluated
314 by Raman and FT-IR spectroscopy. Complete removal of water molecules from dihydrate
315 gypsum makes it more condensed crystalline form (orthorhombic anhydrite) [46] that promote
316 their bioavailability to animal cells. According to Ayurvedic pharmaceuticals, an ideal heating of
317 inorganic minerals is essential for required physicochemical changes during Bhasma formulation
318 [47]. The present finding supports the ancient concept of the Bhasma formulation.

319 Surface charge of particle is an important factor for cellular internalization. It is obvious that the
320 interaction between positively charged particles with negatively charged cell membrane increase
321 cellular uptake [48, 49]. It is interesting to observe from our experiments that gypsum which is
322 near to neutral charge was not capable to induce vacuole formation while neutral charged GB
323 particle induced vacuolation effectively. Therefore, our present study reveals that charge is not
324 an important factor for cellular internalization of GB particle.

325 The possible reason of such internalization of GB particles might be its structural rearrangement
326 that facilitated to recognize cell surface receptors and, thus permeating into cells by receptor-
327 mediated cellular uptake. Receptor-ligand interaction during phagocytosis is well studied,
328 *Fcγ receptors* which recognize particles coated immunoglobulin *G* is the most widely studied
329 example of phagocytosis [24]. However, few studies have been reported on the interaction of
330 particle-bound ligand-receptors of non-phagocytic cells. The life-threatening human pathogen
331 *Staphylococcus aureus* have an ability to internalize in a variety of non-phagocytic cells like
332 epithelial, endothelial, fibroblast, osteoblast cells etc. The pathogen uses $\alpha 5\beta 1$ integrin receptor,
333 chaperons and the scavenger receptor CD36 to internalize into target host cells [50]. *Gratton et*
334 *al* [19] revealed that HeLa cell internalized PRINT particle of 1-3 μm by several different
335 mechanisms of endocytosis. However, the extensive study will be needed to understand the
336 interaction between GB particle bound ligand and cell surface receptors, and downstream
337 molecular mechanism.

338 Flow Cytometry images revealed phagocytic cup formation at the time of particle internalization.
339 The phagocytic cup formation is well established by many authors [23, 42, 43]. After particle
340 internalization, the nascent vacuoles appeared around the perinuclear region and a gradual
341 increase in the number and size of vacuoles was observed until much of the cytoplasm occupied
342 by a single or several vacuoles. The nascent vacuole fused with lysosome, transferring lysosomal
343 contents to become phagolysosomes. Our experiment revealed that GB induced vacuolation
344 required V-ATPases. V-ATPase supply high concentration of H^+ leading higher osmotic pressure
345 within vacuoles, and thus resulting in large vacuole due to the influx of water molecules [51-53].
346 V-ATPases are found on the membranes of intracellular vacuolar organelles, like endosomes,
347 lysosomes, and phagolysosomes [25,26].

348 Further flow cytometry analysis of cell lysate indicated that the internalized particles were found
349 to be degraded slowly. The degradation was also clearly confirmed by time lapse microscopy
350 targeting single cell with intravacuolar GB particle. The intracellular degradation of GB particles
351 observed in endocytic vacuoles was due to its acidic environment. In our study, the vacuolar
352 acidic environment was broken with the treatment of BFA1 and CQ, resulting inhibition of
353 vacuole progression and following particle degradation. Increasing of luminal pH of vacuole also
354 blocks the fusion of phagosomes and late endosomes [28]. However, BFA1 and CQ induce
355 autophagic vacuole in cells have been well studied [54,55]. In our study, we found that GB

356 particle was degraded in cell free environment having pH 5-6 which mimics vacuole-lysosomal
357 pH. We calculated time taken for bhasma particle degradation in cell free environment and
358 vacuolar lumen of 3T3L-1 cells. We found that in cell free condition, particle digestion was
359 taken 10 h at pH 5, 24 h at pH 5.5 and more than 24 h at pH 6 whereas, in cellular system it took
360 less than 24 h for complete degradation. In the video, targeting single cell with intravacuolar
361 particle, we also found that the bhasma particle degraded at 8 h. These phenomena indicated that
362 the acidic environment inside the vacuole might be the reason for particle degradation. However,
363 lysosomal enzymes are activated in the acidic pH to break down various cellular and non-cellular
364 components. Lundborg et al 1984 [56] suggested that metal particles dissolve in the alveolar
365 macrophages might be due to the reduced pH in the phagosomes. Therefore, our findings
366 indicated that lysosomal enzymes might have no role in the GB particle degradation.

367 GB induced vacuolated cells was normal in proliferation which was evident by MTT and cell
368 migration assay. These findings clearly indicated that GB does not cause any significant effect
369 on cell toxicity as well as cell proliferation. The vacuolation is the sign of cell death in most of
370 the cases by triggering apoptotic or non-apoptotic pathway [57-62]. Initially, it was assumed that
371 the accumulation of vacuoles in cell cytoplasm hampers cell functions and cause cell death. The
372 mechanisms of cell death have been studied by many authors, mainly not due to vacuolation but
373 through disruption of mitochondria, endoplasmic reticulum, Golgi apparatus, and endo-
374 lysosomal system [8, 59, 63-65]. Moreover, the cell protects itself from a toxic substance by
375 developing vacuole to separate from the cytoplasm. In this connection vacuolation in cells is an
376 adaptive physiological response, presumably for damage limitation. Where damage limitation
377 fails, cells usually die quickly. In our experiment, post treatment of BFA1 and CQ in vacuolated
378 cells by GB showed a significant cell death compared to GB and BFA1/CQ control. Therefore, it
379 is confirmed that the vacuole inhibitors stopped the vacuolar function by increasing pH in
380 vacuolar lumen and thereby inhibiting further vacuolar progression. The viability loss could
381 include failure to degrade GB particle or persistent of faulty vacuolar organelles. Whereas only
382 GB treated cells survive with active and complete vacuolar process. Also BFA1 and CQ disrupt
383 autophagy flux, leading accumulation of autophagosomes in cells [54, 55]. In the post BFA1/CQ
384 treatment experiment, it can be concluded that excessive accumulation of autophagosome and
385 defective vacuolar function is wasteful process to the cell, there by exerting cell toxicity. In our
386 experiment, the LAP-like LC3 lipidation is activated in GB induced vacuolated cells evidenced

387 by accumulation of LC3-II on vacuolar membrane whereas LC3 localized in nucleus in untreated
388 cells. This result is strongly supported by the up regulation of LC3 in cell lysate. During vacuolar
389 progression the nuclear LC3 distributed in cytoplasm as soluble LC3-I which in turn conjugated
390 with lipid forming LC3-II and recruited to vacuolar membrane [66-68]. The BFA1 treatment in
391 vacuolated cells disrupts LC3-II accumulation on the vacuolar membrane. Florey et al 2015 [53]
392 suggested that LC3 lipidation is completely blocked by BFA1 treatment. Based on previous
393 report and our current finding, it can be concluded that LAP like function is essential for
394 vacuolated cell survival. Our study indicated a direct evidence of survival strategy of vacuolated
395 cell. The survival response of GB induced vacuolated cells from ions toxicity might be correlated
396 with controlled and sustained release of Ca^{++} and SO_4^- due to slow dissociation of GB particle
397 inside vacuole. Perhaps cells get advantages by utilizing the ions for better physiological
398 purpose. Also, cell restored its normal morphology by decreasing vacuolar volume which is
399 associated with cellular protection from potential damaging swelling pressures.

400 GB is a traditional medicine used in India since a long time for treating mainly digestive
401 impairment, acid-peptic disorders as well as bone-related disorders. Further research will be
402 needed using *in-vivo* as well as *in-vitro* diseases model to evaluate its pharmacological
403 mechanism of action. Also, GB induced vacuole formation in mammalian cells (non-phagocytic)
404 will be a powerful model to study vacuole biogenesis and will undoubtedly identify novel
405 molecular players by manipulating gene of interest.

406

407 **Materials and methods**

408 **Preparation of Godanti Bhasma**

409 For the preparation of GB, initially, raw gypsum was coarsely powdered and washed with warm
410 water. Then, it was suspended in sufficient quantity of lemon (*Citrus limon* L. Osbeck) juice and
411 then subjected to moderate heat (~80 °C) for 90 min. The obtained material was washed with
412 warm water, dried and used for further process. In the next step, the purified powder was placed
413 in an earthen crucible and subjected to Gaja Puta (classics nomenclature used for the quantum of
414 heat) heating in a muffle furnace at ~800 °C for 30 min, and then allowed for self-cooling. The
415 obtained material was further impregnated with Aloe vera (*Aleo barbadensis* Mill.) juice and
416 subjected to another Puta. Finally, white colored Godanti Bhasma is obtained [32].

417

418 **Characterization of raw gypsum and Godanti Bhasma (GB)**

419 In this study, initially Confocal Raman (WITec Confocal Raman system; model: Alpha300
420 series), and Fourier-transform infrared (FT-IR) spectroscopic techniques were used to
421 characterize the changes in raw gypsum and GB. Raman images and spectra were recorded using
422 ultra-high-throughput *spectrometer* (UHTS) equipped with a charge-coupled device (CCD)
423 camera, diode laser used for 532 nm excitation, and microscope (Zeiss 100x air objective with
424 numerical aperture 0.9). The FT-IR measurements were obtained at room temperature using
425 AGILENT spectrometer (model: CARY630) equipped with ATR cell attached with Micro Lab
426 PC software. The data was recorded three times from three different set of samples for both
427 experiments.

428 Further, the surface morphology and particle size distribution of GB sample were observed by
429 Field emission scanning electron microscopy (FESEM) (TESCAN; model: MIRA3) technique.
430 For this sample was anchored on the sample holder, and morphology was probed on selected
431 points to determine elements with the help of detector inbuilt with energy dispersive X-ray
432 analyzer (EDX) (Rigaku; model: XFlash 6I10) at 0-10 keV. Total three different set of particles
433 were examined during scanning. Histograms of particle size distribution were made on three
434 independent FESEM images.

435 Zeta potentials were measured to evaluate electrochemical changes at the microparticle surface due to
436 the thermal transformation from gypsum to GB. For this samples were suspended in distilled water as

437 well as 10% FBS and surface charged were assessed by zeta sizer nano (Malvern Panalytical, UK,
438 ZS90). Five samples of each set were recorded.

439

440 **Cell Culture**

441 Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), Antibiotics, Trypsin-
442 EDTA solution were obtained from Thermo Fisher, MA USA. Cell lines (3T3-L1, L6, Neuro 2a,
443 HeLa, HCT-116, A549, MDA-MB231, and MCF7) were procured from National Centre for Cell
444 Sciences (NCCS), Pune, India. The cell was cultured in DMEM (GIBCO BRL, Grand Island,
445 NY, USA) supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100
446 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in a humidified atmosphere of
447 5% CO₂ at 37 °C. The cells were dissociated with Trypsin-EDTA solution. The stock cultures
448 were grown in 25 cm² culture flasks, and experiments were carried out in 96- and 6-well plates
449 (Tarsons India Pvt. Ltd., Kolkata, India).

450

451 For cell culture experiment, GB powder (100 mg) was suspended with complete DMEM media
452 (1 ml supplemented with 10% FBS) by vortexing, and leave the tube on a stand for 1 min to
453 settle down larger particle, serial dilution was done using 500 µl of GB suspension. All cell
454 culture experiment (except dose-response experiment) was conducted using 5th dilution of GB
455 suspension.

456

457 **MTT assay**

458 Cell viability was determined using MTT assay in 3T3L1. Cells were seeded (7500 cells/well) in
459 96-well plate and incubated for 24 h. After 24, 48 and 72 h of incubation in presence of GB with
460 serial dilutions (0 – 2.5 mg/ml), the culture medium of each well with or without extract was
461 removed completely from the assay plates and replaced by fresh culture medium (100 µL). MTT
462 (Thiazolyl Blue Tetrazolium Bromide) solution (10 µL of 5 mg/mL), (Thermo Fisher, MA USA)
463 was added into each well to achieve a final concentration of 0.45 mg/mL before incubated for 3 h
464 at 37 °C. After 3 h, the culture medium with MTT was carefully removed followed by addition
465 DMSO (100 µL) (Himedia, India) to dissolve formazan crystals, and then incubated for 1 h
466 before recording the optical density (Envision plate reader, California, USA) at 540 nm. Cell
467 viability test was also performed in six different cell lines (Neuro 2a, A549, MDA-MB231,

468 MCF-7, HCT-116, and HeLa) at 24 h of culture. The results are presented as the mean \pm SD
469 (n=6).

470

471 **Cell migration assay**

472 Cells were seeded in a 6-well plate (0.5×10^6 cells/well), incubated up to 100% confluent. Cells
473 were treated with and without GB. The monolayer of cells was scratched (3 scratches) with a
474 pipette tip after 12 h of treatment, and cells were imaged at 0, 4, 8h post-scratch. The cell-free
475 areas per treatment group were used for analysis.

476

477 **Caspase assay**

478 The Caspase-3 colorimetric assay was also conducted according to manufacturer instructions
479 (Sigma-Aldrich, MO, USA). 3T3-L1 cells were treated with Bhasma for 0, 2.5, 5, 10, and 24 h.
480 The concentration of the p-nitroanilide (pNA) released from the substrate was calculated from
481 the absorbance values at 405 nm. Three samples were done for each time points.

482

483 **Crystal Violet staining**

484 Crystal Violet (High Media, India) staining in cells was done for microscopic imaging. Treated
485 cells were washed with 1X PBS, fixed with formaldehyde (10%) for 15 min. After fixation, cells
486 were washed with water and stained with 0.5% (w/v) crystal violet (25% (v/v) methanol) for 25
487 min, subsequent washing the cells with water until no color was eluted, and images were taken
488 by Bright Field microscope (PrimoVert. Zeiss, Germany).

489

490 **Neutral Red staining**

491 Relative vacuolation was quantified based on the uptake of the Neutral Red dye (High Media,
492 India) in mammalian cells (Kannan et al. 2014). The experiments were performed in 96-well
493 plates, neutral red (100 μ l of 0.5 mg/ml) was added in each well, incubated for 4 h in CO₂
494 incubator, washed 3 times with PBS, eluted the neutral red by adding destaining solution (100 μ l
495 containing 50% dehydrated ethanol, 49% deionized water and 1% glacial acetic acid). Neutral
496 Red uptake was determined using a microtiter plate reader (EnVision Multimode plate reader,
497 Perkin Elmer, USA) to measure the absorbance at 540 nm.

498

499 **Acridine Orange (AO) staining**

500 Cells were grown on glass coverslips, treatment was done for a specific period, the cells were
501 incubated with acridine orange (1 mg/ml) (High media, India) for 15 min at 37 °C followed by 3
502 PBS washes, and then immediately observed under a fluorescence microscope (Mantra,
503 PerkinElmer).

504

505 **Processing of Cells and Its Characterization by FESEM and EDX**

506 Cells were cultured in culture disc up to 70% confluency, GB treatment was done for 18 h after
507 treatment cells were washed 5 times with PBS to remove extracellular particle, cells were
508 trypsinized, centrifuged (1000 rpm for 10 min) to make pellet. Cells were re-suspended in PBS,
509 re-pellet, and replaced with formalin (10%) to fix for 1 h. Cells were replaced sequentially with
510 70, 95, and 100% ethanol for 1 h each. Treated cells were replaced with xylene (2 times, 1 h) and
511 paraffin wax (2 times, 90 min), refrigerated to set the wax. Wax blocks (containing the cell
512 pellet) were sectioned and selected section was placed on round cover glass, which eventually
513 treated with xylene to remove paraffin and washed (3 times, 5 min each) with PBS. For SEM of
514 fixed cells (without section), cells were also grown on the cover glass, treated with GB particle,
515 fixed with 10% formalin (30 min) and washed 3 times. The processed cells were further
516 characterized through FESEM and EDX stated above. The whole procedure was done from three
517 different samples. Further images of FESEM and EDX was done from each samples.

518

519 **Cell Preparation for Flow Cytometry**

520 Cells were seeded (0.2×10^6 cells/ well) in 12-well plate and cultured for 24 h, GB treatment was
521 done at different time points (30 min, 1h and 12h), washed with PBS (5 times) to removed extra
522 particles, trypsinized and washed again in PBS. The cells were run in Flow cytometer (Amnis®
523 Imaging Flow Cytometers, Millipore, USA). Individual cell images were collected from Flow
524 cytometer image library and analysis was done.

525 For particle analysis, cells were cultured (0.2×10^6 cells/ well) in 12-well plates for 24 h,
526 treatment of GB was done at different time period (1h, 7h, 14h and 24h), culture media were
527 collected in 1.5 ml tubes, the cells were washed with PBS (5 times) to remove particles present
528 in outside of cells, and 500 μ l RIPA (20 mM Tris-HCl pH-7.5, 150 mM NaCl, 0.1% Triton X-
529 100, 1% sodium deoxycholate) buffer was added to lyses cell completely. The culture medium

530 and lysed cell samples containing particle were run in Flow cytometer. Particles suspended in
531 culture media and cell lysate samples were analyzed by the flow cytometer software IDEAS
532 (Amnis Corporation, WA, USA). The two independent experiments were done.

533

534 **Time Lapse Microscopy**

535 Cells were grown up to 70% confluency in petridish. GB suspension was added into the culture
536 medium and placed the culture dish on stage of imaging microscope (JULI smart fluorescent cell
537 analyzer, Seoul, Korea). The images were captured at every 5 min for overnight. The whole set
538 up was kept in a CO₂ incubator. Movie was made using captured images by software Image J.
539 The time lapse microscopy was done three times.

540

541 **Different pH Treatment of GB Particles and Turbidity Test**

542 Turbidity is the cloudiness or haziness of a fluid containing particles. 1X PBS solution (2.2 mM
543 KCl, 1 mM Na₂HPO₄, 140 mM NaCl) at different pH (7.5, 7.0, 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0)
544 was made. GB powder (100 mg) was suspended in water, mixed by vortex, and kept in a stand
545 for 1 min to settle down larger particles. Particle suspension (10 µl) was added in PBS (200 µl)
546 of respective pH (n=6) in 96-well plate. The measurement of turbidity was done at different time
547 points (15 min, 90 min, 3 h, 6 h, 12 h, and 24 h) in envision plate reader at 580 nm. The
548 absorbance are plotted (mean ± SD). After 24 h of treatment, images of particle were taken by
549 light microscope (Zeiss, Primovert).

550

551 **Immunocytochemistry**

552 After treatment, cells were washed with PBS (2 times) and fixed with 4% paraformaldehyde for
553 15 min. Following three further PBS washes, cells were permeabilized with 0.2% Triton in PBS
554 for 10 min. Cells were blocked in blocking buffer (1% BSA, PBST) for 1h at room temperature
555 and incubated with primary antibody LC3A/B (Thermo Scientific, USA) overnight at 4 °C. Cells
556 were again washed with PBS (3 times, 10 min each), incubated with secondary antibody Goat
557 Anti Rabbit IgG H&L dylight 488 (Thermo Scientific, USA) for 1 h, and then followed by PBS
558 wash (3 times, 10 min each). Only secondary antibody (no primary ab) in treated cells was used
559 as a control. Cells were treated with DAPI (3 g/ml) in PBS for 5 min, wash again with PBS (3
560 times, 10 min each), Slides were mounted with 50% glycerol. Microscopy was done with

561 fluorescence microscope (Mantra, Perkin Elmer, USA). The immunocytochemistry was done
562 three times.

563

564 **Western blotting**

565 Cells were washed with PBS, scraped into ice-cold RIPA (150 mM NaCl, 50 mM Tris-HCl, 1%
566 Triton-X-100, 0.1% SDS, 0.1% sodium deoxycholate) buffer and lysed for 10 min on ice.
567 Lysates were centrifuged for 12 min at 4°C. Supernatants were then separated on 15%
568 polyacrylamide SDS-PAGE gels and transferred to a PVDF membrane. The membrane was
569 blocked in TBST (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1% Tween 20) + 3% BSA and
570 incubated overnight at 4°C with primary antibody LC3A/B diluted in blocking buffer. Blots were
571 incubated with HRP conjugated to secondary antibody (Thermo Scientific, USA) and protein
572 detected using enhanced chemiluminescence (Thermo Scientific, USA). The blot was striped
573 with striping buffer (0.1M glycine 0.1% SDS 1% Tween20, pH to 2.2) and reprobed with
574 GAPDH antibody.

575

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584

585 **Conflict of Interest**

586 The authors declare no conflict of interest.

587

588 **Author contribution**

589 S.K.D. conceptualization, conducted Particle characterization, cell culture experiments,
590 immunostaining, western blot, analyzed all data, manuscript writing, reviewing and supervised
591 overall studies; A. J. conducted the immunocytochemistry, Flow Cytometry study, analyzed the

592 data and manuscript writing; V.S. Prepared Bhasma, Particle characterization (particle
593 SEM+EDEX) manuscript writing and reviewing; L.B. assisted cell experiment, cell staining
594 procedure and western blot; N.D. performed particle charge, N.S. performed FTIR; S.S. made
595 movie from pictures of time lapse microscopy; D.M. helped for immunocytochemistry; K. J.
596 prepared cell sections for SEM; A.B. helped for Ayrvedic conceptualization, S.D. Checked all
597 data, reproducibility and review manuscript.

598

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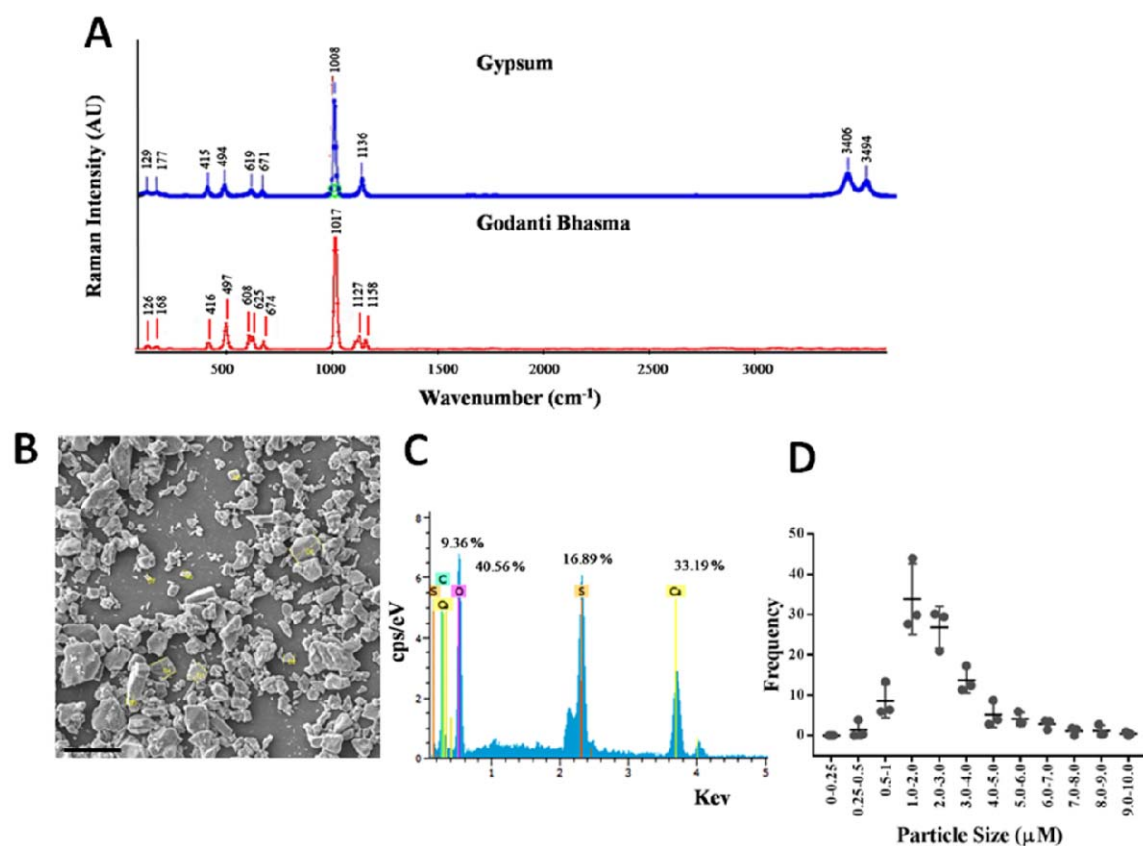
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776 Figures and Legends



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779 Fig. 1: Characterization of gypsum and Godanti Bhasma

780 (A) Raman spectra of Gypsum and Godanti bhasma. The Godanti bhasma (GB) is anhydrous
781 calcium sulphate produced by thermal transformation of gypsum. Raman spectra showing two
782 water molecules were disappeared in Godanti Bhasma. The spectral shifts were observed clearly
783 between the two compounds. (B) Field Emission Scanning Electron Microscopy (FESEM) image
784 showing irregular particles sizes, Scale bar = 20 μm. (C) Compositional analysis and weight
785 percentage (O, Ca and S) of particles calculated from Energy-dispersive X-ray spectroscopy
786 (EDX). (D) Histograms showing particle size distribution based on three independent FESEM
787 images (mean ±SEM).

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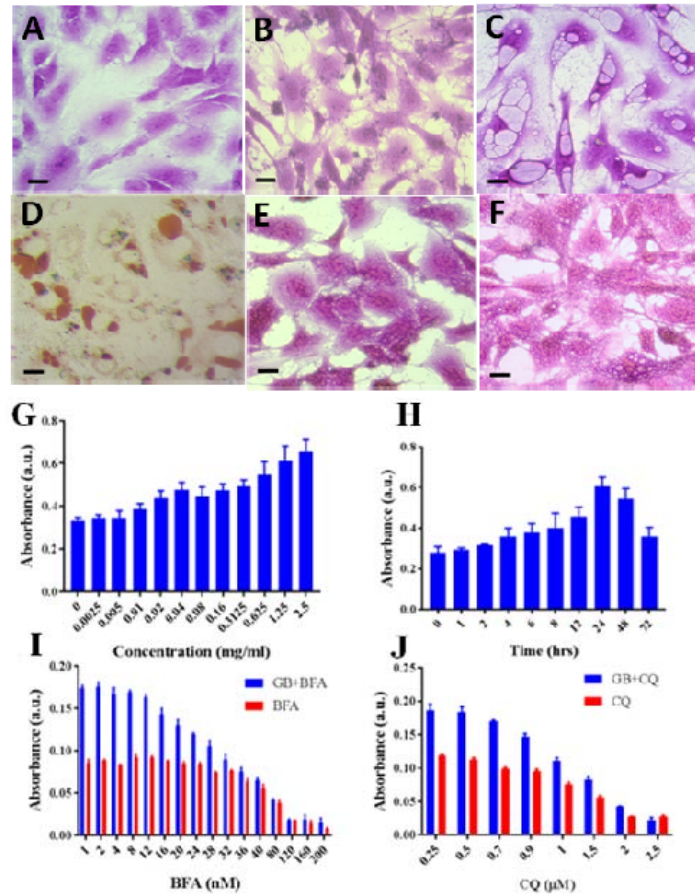
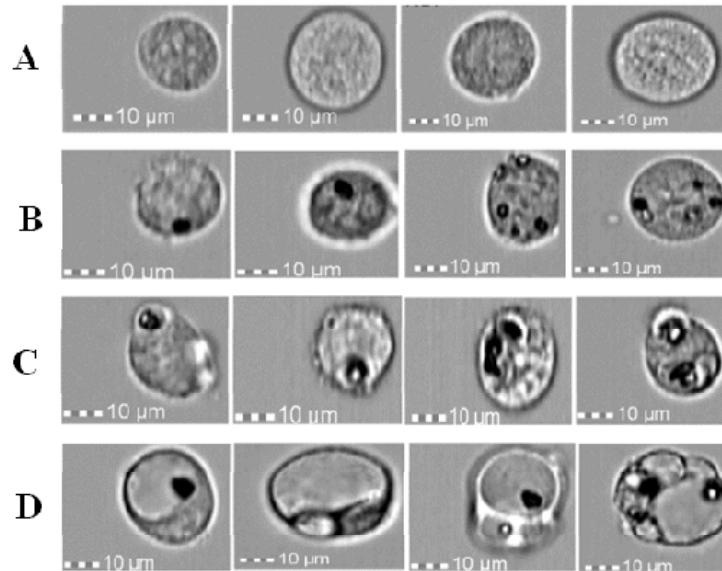


Fig. 2: Vacuole biogenesis in 3T3-L1 cells. (A) Untreated cells, (B) Gypsum treated cells showing no vacuoles, (C) Godanti Bhasma treated (0.15 mg/ml) cells showing vacuolar structure in the cytoplasm of cells; (D) GB treated cells stained with Neutral red showing acidic pH inside the vacuole. (E) Co-treatment of cells with GB (0.15 mg/ml) and BFA1 (100 nM), (F) Co-treatment of cells with GB (0.15 mg/ml) and CQ (2 μM). Both vacuole inhibitors suppress vacuolation. Cells were stained with Crystal violet and images were captured by a bright field microscope. (Scale bar = 20 μm) (G) The dose response experiment showing vacuolation increased with increasing concentration of GB treatment, (H) GB induced vacuolation increased with increasing times upto 24 h, (I) Cells were treated with GB+BFA1 and BFA1 alone. BFA1 prevents lysosome and vacuolar pH by blocking V-ATPase on their membrane. (J) Treatment of cells with GB+CQ and CQ alone. CQ trapped inside vacuole and increased vacuolar pH. GB was used in 0.15 mg/ml, whereas BFA1 and CQ were used in different concentrations. The results (G-J) are presented as the mean ± SD (n=6).

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833 **Fig. 3: Flow Cytometry cell images at different time points after GB treatment.** Cells at
834 different time points (30 min-12 h) were trypsinized and run in Flow cytometry, Single-cell
835 images were collected from Amnis Flow cytometer Image gallery at different time points. (A)
836 Untreated cells, (B) In 30 mins, cells showing particles are attached to the cell surface, (C) At 1
837 h, cells showing GB particle internalization by membrane invaginations or outgrowth, (D)
838 Treated cells at 12 h show large vacuole containing particle.

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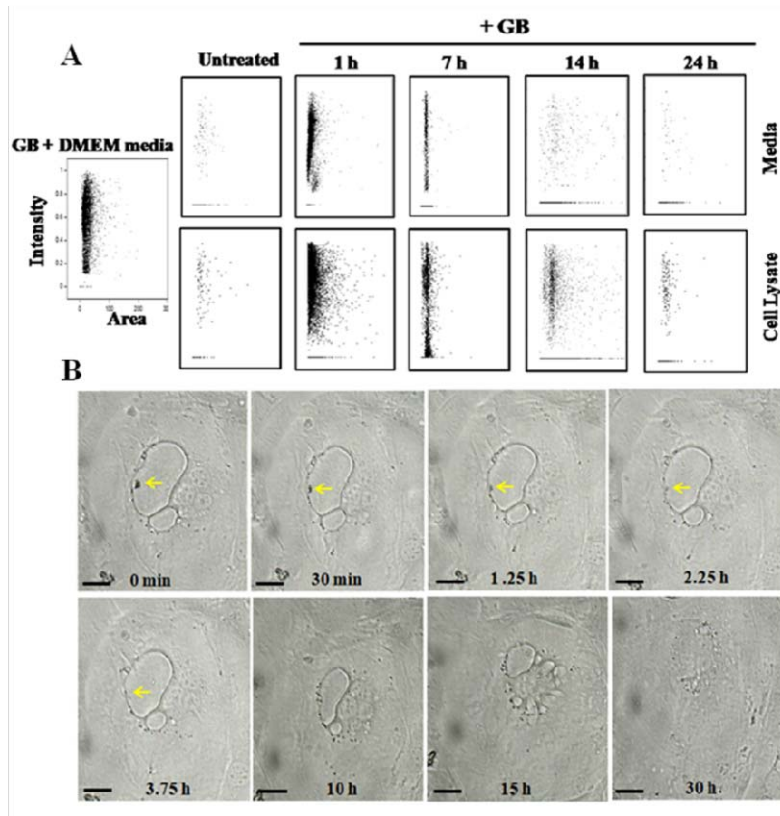


Fig. 4: GB particle degradation in 3T3-L1 cells. (a) Flow Cytometry Analysis of GB Particle in Cell Lysate and Culture Medium. (X-axis-brightfield area; Y-axis-bright field aspect ratio of intensity). GB particles were allowed to incubate with 3T3-L1 cells at different time periods (1, 7, 14 and 24 h) at 37 °C to internalize and following degradation. Complete DMEM containing GB particles and Untreated cells were used as control. After incubation culture media were separated from cells. Cells were lysed with RIPA buffer to release the particles; the gate was set on particles. Non-internalized particles were in culture media. Both particles in culture media and cell lysate were analyzed simultaneously by flow cytometer software (IDEAS). (b) Time-Lapse Microscopy of a single cell showing particle degradation inside the vacuole. Microscopic images of a single cell (GB treated) were captured and GB particle was marked with an arrow (yellow) inside the vacuole. The particle was degraded overtime and following vacuolar turnover was also observed restoring normal morphology of cells. Images of the cell were captured in the respective times. Scale bar = 20 μ m.

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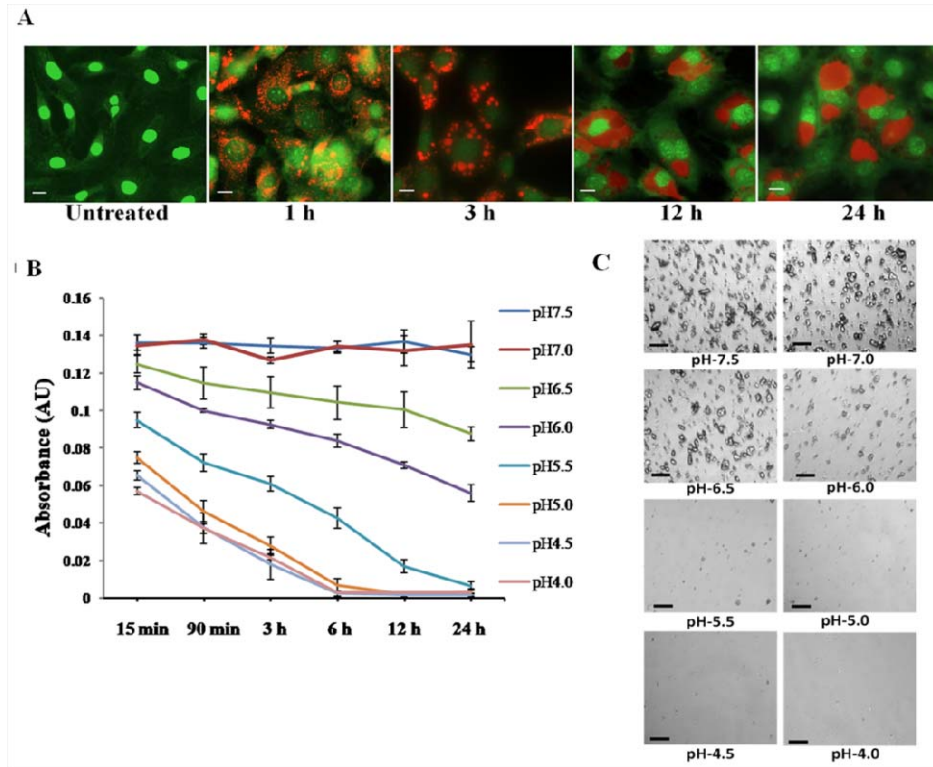


Fig. 5: Dissolution of Bhasma particle in vacuolar pH (A) Time course experiment (1h, 3h, 12h and 24 h) of GB treatment in cells. After the treatment, cells were treated with Acridine Orange showing stained (orange-red) vacuole that maintained acidic environment. Acridine orange crosses into lysosomes and becomes protonated resulting orange-red colored vacuole in treated cells, whereas in untreated cell it emits green color. The nucleus of each cell was stained as green. **(B)** The GB particles were treated in PBS buffer with different pH (4-7.5) and at different time intervals (15 min to 24 h). Turbidity test was done after specific times. At physiological pH (7 and 7.5) GB particles were not soluble, whereas the particles were gradually soluble with decreasing pH (Lysosomal/vacuolar pH; 6.5 to 4.5). The pH 5 showed the lowest solubility which is mimicking acidic vacuolar environment. The results are presented as the mean \pm SD (n=6). **(C)** Microscopic images of GB particles at different pH after 24 h.

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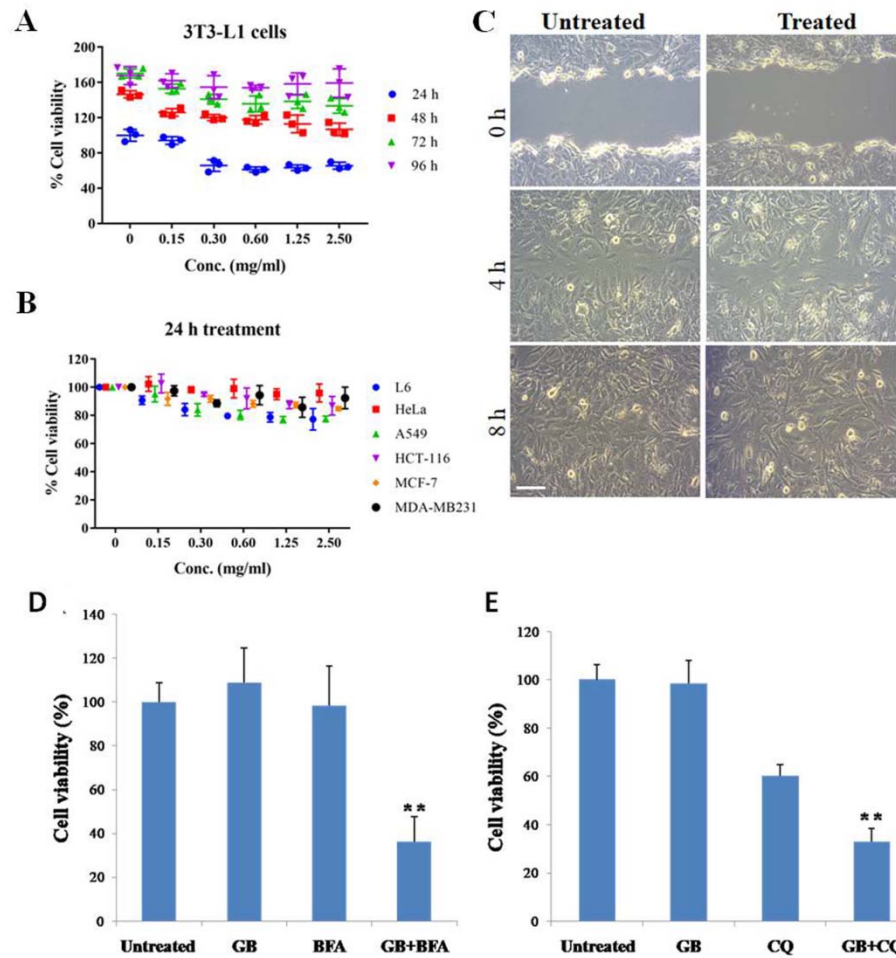
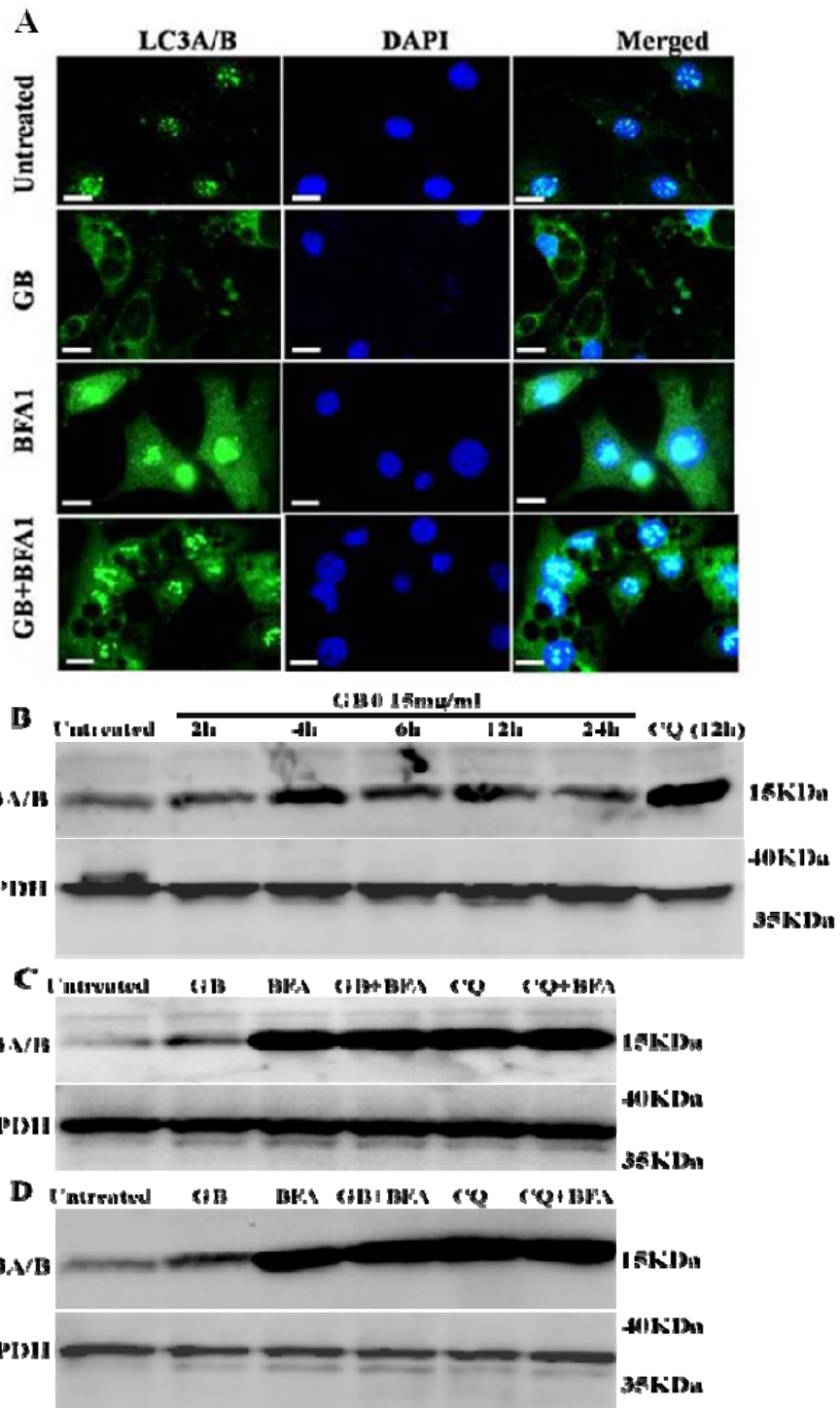


Fig. 6: Effect of Godanti Bhasma on Cell Viability and Proliferation. (A) Cell viability in 3T3-L1 at different time points (24, 48, 72 and 96 h) at concentration ranges from 0-2.5 mg/ml using MTT assay (B) Cell viability in L6, Neuro 2a, HeLa, MDA-MB231, A549, HCT 116 and MCF-7 at concentrations vary from 0 – 2.5 mg/ml after 24 h of treatment using MTT assay, (C) Scratch assay in Bhasma treated 3T3L1 cell at different time points (0, 4 and 8 h). The cells were viable and proliferation was normal. , Scale bar = 50 μ m. (D) Loss of viability of GB induced vacuolated cell by post treatment of Vacuole inhibitor BFA1 with controls, and (E) Loss of viability of GB induced vacuolated cell by Post treatment of CQ in GB treated cells with controls. A significant toxicity of GB induced vacuolated cells was observed by the post treatment of BFA1/CQ compared to GB and BFA1/CQ controls. The concentration of GB, BFA1 and CQ were 0.3 mg/ml, 100 nM and 1 μ M respectively. $**P < 0.01$. The results are presented as the mean \pm SD (n=6).

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969 **Fig. 7: GB activated LAP like function.** (A) Fluorescence image (40X) of Immuno-staining
970 with LC3 A/B antibody showing LC3 expression in 3T3L1, LC3 expressed within nucleus in
971 untreated cells, whereas in GB treatment, LC3 expression was found in membrane/ periphery of
972 vacuoles indicating presence of LAP like function. LC3 expression in BFA treated cells showing
973 LC3 expression in whole cell, and In GB+BFA, the post treatment of BFA was done in GB
974 induced vacuolated cells, showing absence of LC3 (LC3-II) accumulation on vacuolar membrane
975 indicating LAP like function was suppressed, whoever expression was present in whole cell. (B)
976 The western blot showing expression of LC3 in 3T3L1 cells in different time points showing
977 LC3 expression was in steady state compared to untreated and CQ treatment. Untreated cells was
978 showing very less expression whereas CQ High expression, (C) Showing LC3 expression by the
979 treatment of GB with or without autophagy inhibitors (BFA and CQ), BFA and CQ inhibited of
980 vacuolar function as well as autophagy flux, and (D) Neuro2a experiment same as of (C).
981 Vacuole inhibitors BFA and CQ abolished the LAP function in cells by inhibition of vacuolar
982 function as well as autophagy flux. Scale bar = 20 μ m.
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