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Aqueous extract of *Calamus rotang* as a novel immunoadjuvant enhances both humoral and cell mediated immune response

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ABSTRACT

Introduction: Search for new adjuvants for human vaccines has become an expanding field of research in the last thirty years for generating stronger vaccines, capable of inducing protective and long lasting immunity in humans. The objective of this study was to investigate the immunoadjuvant activity of aqueous extract from the leaves of *Calamus rotang* using phosphate buffered saline (PBS, pH 7.2) against hepatitis B vaccine containing surface antigen (HBsAg; 20 µg/mL).

Methods: In this research qualitative study was evaluated in order to determine the presence of secondary metabolites and further confirmation of these metabolites through high performance thin layer chromatography (HPTLC) and identification by liquid chromatography mass spectrometry (LC-MS). In addition, indirect Elisa was performed using HBsAg as coating antigen and this aqueous extract showed anti-HBsAg titre at higher doses as compared to standard and control. In continuation of these studies, Swiss mice were immunized subcutaneously on day 0 with HBsAg (20 µg/mL, 100 µL) and collect splenocytes on day 4 for splenocyte proliferation assay (ex vivo studies; again exposed with HBsAg) and estimation of Th1 (IFN-gamma and tumor necrosis factor [TNF-α]) cytokines from cell culture supernatant.

Results: The aqueous leaves extract of *C. rotang* showed dose dependent enhancement in antibody titre and proliferation at higher doses ($P<0.01$) with respect to HBsAg. In addition, this aqueous extract also showed improvement in Th1 (IFN-gamma and TNF alpha) cytokines at higher doses ($P<0.01$) from cell culture supernatant as compared to standard HBsAg.

Conclusion: *Calamus rotang* has additive adjuvant activity against hepatitis B vaccine antigen containing alum and may help to raise antibodies against HBsAg under challenging administration regimen and might be a potent vaccine adjuvant.

Implication for health policy/practice/research/medical education:
Aqueous extract of Calamus rotang may help to raise antibodies against HBsAg under challenging administration regimen and could be a potent vaccine adjuvant.

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Introduction

Despite the success of current vaccines, there is an urgent need or requirement of adjuvants for vaccines against various infectious diseases (1,2). Recently, one of the approved adjuvants for human use i.e. alum, enhanced only humoral immune response and poorly elicited cell mediated immunity (3). Currently many plant based immunomodulators, i.e., stimulatory or immunosuppressive compounds or molecules, are reported or still in clinical trials and these plant based candidates tried to use as adjuvants against specific protein antigen (4,5). In other words, plant based immunomodulators are under evaluation as newer and safer adjuvants (4,5). In view of credibility of plant-based adjuvants with numerous supporting and related immunological bioactivities, it seems worthwhile that the directional approach may help grant potential protection to immune system in combination with vaccines. One of the most familiar examples of plant based adjuvants i.e., QS21 from *Quillaja saponaria*, showed significant immunomodulatory activity (6,7). The ability of plant-based

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vaccine adjuvant to deliver sufficient antigen to induce protective immune responses is now well established for a wide range of antigens. Today's development of novel vaccines stresses the need for plant-based adjuvants that are inexpensive, easily administered and capable of being stored and transported without refrigeration (8,9). Without these properties, developing countries find it difficult to adopt vaccination as the central strategy for preventing their most devastating diseases. A promising approach is the production of vaccines containing plant-based adjuvants.

High performance thin layer chromatography (HPTLC) is a powerful method used for qualitative and quantitative analytical tasks. Applications of HPTLC such as identification and quantitation of bioactive phyto-constituents, impurities, process optimization, monitoring and standardization have been demonstrated (10). It is also reported to achieve excellent separation, validation of methods for herbal and botanical dietary supplements, nutraceuticals, various traditional healthcare systems (western medicines, Ayurveda) (11). Likewise liquid chromatography mass spectrometry (LC-MS) is believed to be the easiest, accurate and appropriate technique for phytochemical profiling. This system presents synergistic approach of good separation power of liquid chromatography with excellent identification capacity of mass spectrometry to identify various phytochemicals present in the aqueous extract. In order to achieve this objective, one of proposed adjuvant candidate i.e. aqueous leaves extract of *Calamus rotang* showed various immunopharmacological activities (12-14). In the present study, our group evaluated the immunoadjuvant activity of aqueous leaves extract of *C. rotang* against hepatitis B surface antigen (HBsAg).

Materials and Methods

Plant material

Assemblage of fresh plant leaves of *C. rotang* were collected from Vidya Pratishthan's garden in Baramati, Maharashtra, India. In this study, 5 g of fresh plant leaves were taken separately, macerated in liquid nitrogen to prepare fine powder and then dissolved in phosphate-buffered saline (PBS). Collect the supernatant in the form of filtrate after centrifugation and then proceed for the estimation of secondary metabolites (qualitatively/quantitatively) and tested for immunoadjuvant activity.

Qualitative estimation of secondary metabolites

In an effort to estimate the presence of secondary metabolites in aqueous leaves extract qualitatively various tests were used for the presence of terpenoids, flavonoids, glycosides and phenolics. Qualitatively, this aqueous extract showed the presence of terpenoids, flavonoids, glycosides and phenolics but no alkaloid. In addition, HPTLC analysis was carried out for phenolics and glycosides as shown in Figure 1. For phenolics, leaves sample was extracted with 70% ethanol. The plant was extracted at 60°C, dried and dissolved in methanol. The mixture was centrifuged at 3000 rpm for 5 minutes. The supernatant was used for sample application. In case of glycosides, the leaves sample was extracted with 70% ethanol (25 mL). The extract was kept in rotary shaker (120 rpm) for 8 hours. Lead acetate was added to the filtrate based on the volume and centrifuged at 5000 rpm for 10 minutes. The supernatant was further centrifuged by adding 6.3% Na_2CO_3 (3 mL) at 10 000 rpm for 10 minutes (approximately for 20 mL filtrate 3 mL of Na_2CO_3 was added). The retained supernatant was dried, re-dissolved in chloroform and used for sample application.

LC-MS analysis and specification

All MS acquisitions were performed in the positive electrospray ionization mode. The capillary voltage, cone voltage, fragmentor voltage were 4 kV, 45 V and 170 V, respectively. The gas temperature was set at 325°C. Data was acquired at scan rate of 3 Hz in mass range 100-100 *m/z*. Further data was analysed with MassHunter qualitative software and METLIN database.

LC was performed using Agilent 1260 Binary LC System having column of Agilent Zorbax SB 18 RRHT column (100×2.1 mm, 1.8 µm) with flow rate - 0.3 mL/min, run time - 30 minutes, injection vol -1 µL and MS at 6540 ultra-high definition accurate mass QTOF LC/MS system using mobile phase A:Water (0.1% formic acid) and mobile phase B: Acetonitrile. The LC gradient conditions were 95% of A and 5% of B for initial 5 to 18 minutes, then 5% of A and 95% of B for 18 to 27.10 minutes, and then 95% of A and 5% of B for final 27.10 to 30 minutes. Acquisition mode for MS with minimum range (*m/z*) was 50 and maximum range (*m/z*) was 1700 whereas scanning rate (spectra/second) was 2.00.

ELISA

Indirect ELISA was performed for detecting immunoadjuvant activity of aqueous leaves extract of *C. rotang* using standard HBsAg (1:1000 dilution) as coating antigen. For these studies, freshly prepared aqueous extract of variable

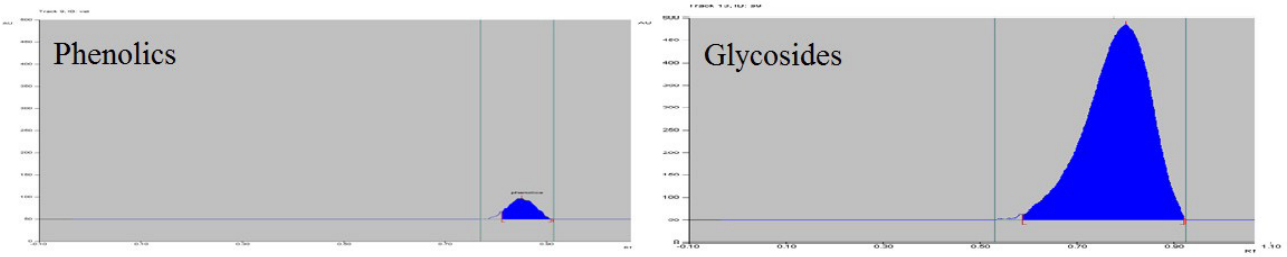

Figure 1. Qualitative HPTLC analysis of *Calamus rotang* for phenolics and glycosides.

doses (2.5–10 mg) were added whereas anti-HBsAg sample was used as standard for estimation of IgG antibody titre. Horse anti-serum was used as secondary antibody and optical density measured at 450 nm (15).

Ex vivo studies (splenocyte proliferation assay)

Immunological studies were conducted under ethical guidelines of animal ethics committee with registration No. 1814/PO/ERE/S/15/CPCSEA.

For these studies, Swiss mice (n = 5; sex- female; weight 18-22 g) were selected and immunized HBsAg (100 µL) subcutaneously on day 0 and day 4, splenocytes were isolated aseptically from the abdominal cavity of mouse. In this assay, splenocytes (10^5 cells/well; 100 µL) were taken in 96 well plate (flat bottom tissue culture plate, Griener) in presence of HBsAg (20 µg/mL; 10 µL) for 48 hours incubation (in carbon dioxide incubator) along with variable doses of aqueous leaves extract of *C. rotang* in a final volume of 0.2 mL.

After incubation, MTT (2.5 mg/mL; 10 µL) solution was added in 96 well plates and again incubated for another 3-4 hours. Thereafter, centrifuging the plate, the supernatant (for estimation of cytokines i.e., IFN-gamma and tumor necrosis factor [TNF-α]) was collected and formazon crystals settled at the bottom were dissolved in dimethyl sulphoxide and the optical density was measured at 570 nm (5,16).

Splenocyte cell culture supernatant containing HBsAg along with variable doses of aqueous leaves extract were collected after 48 hours incubation for the estimation of Th1 (IFN-gamma and TNF alpha) cytokines and performing Sandwich ELISA. This experiment was performed as per the manufacturer's instructions (BD Biosciences) (5,16).

Statistical analysis

The difference between control and treated groups (aqueous leaves extract) of *C. rotang* was determined by one-way analysis of variance (ANOVA) test (Boniferroni multiple comparison test).

Results

LC-MS analysis

The aqueous extract of *C. rotang* was subjected to LC-MS analysis and compounds were characterized based on their mass spectra, using the precursor ion, fragment ions, and comparison of the fragmentation patterns with molecules described in the literature. The analysis identified the presence of various bioactive phytochemicals such as Gentamicin C1a with retention time (rt) 07.00-07.16 minutes and $(M+H)^+$ ion at *m/z* 432. Netilmicin is an antibiotic of aminoglycoside family showing antibacterial property against wide variety of bacteria. It was eluted at 7.26-7.39 minutes on LC column and $(M+H)^+$ ion at *m/z* 476. Dipyridamole is well known to inhibit blood clot formation, having rt 8.35-8.73 minutes and $(M+H)^+$ ion at *m/z* 487. Arbekacin is an aminoglycoside family antibiotic which shows antimicrobial activity against not only gram positive but also gram negative bacteria. Arbekacin had rt 8.53-8.77 minutes and $(M+H)^+$ ion at *m/z* 553. Artemether, an antimalarial drug is also identified by LC-MS having rt 14.34-14.49 with $(M+H)^+$ ion at *m/z* 281. There were many other bioactive phytoconstituents which were identified using LC-MS, representative of which is shown in Figure 2.

ELISA

ELISA (indirect) results confirmed the immunoadjuvant activity of aqueous leaves extract of *C. rotang* against HBsAg vaccine and showed titre at higher doses as shown in Figure 3.

Splenocyte proliferation assay (ex vivo studies)

The effect of aqueous leaves extract of *C. rotang* on splenocyte proliferation assay in Swiss mice (already immunized with HBsAg on day 0 subcutaneously) model studies as shown in Figure 4. The results showed that aqueous leaves extract showed enhancement in proliferation with respect to HBsAg (challenging dose given) at higher doses. HBsAg vaccine used as standard and showed enhancement in proliferation as compared to control.

Th1 cytokines

The effect of aqueous extract on Th1 (IFN-gamma and TNF alpha) cytokines from splenocyte cell culture supernatant is shown in Figure 5. The results showed that aqueous extract enhanced the Th1 cytokines at higher doses as compared to control. In comparison with these two cytokines after estimating from splencoyte cell culture, the IFN-gamma was more effective and released in higher quantity as compared to TNF alpha in aqueous leaves extract of *C. rotang*.

Discussion

The development of plant based adjuvant for vaccine antigens are essential for treating various infectious diseases. However, most of the adjuvants are failed to elicit cell mediated immunity during product development. This study was an effort to search for those adjuvant candidates i.e., plant based that are responsible for inducing both humoral and cell mediated immune responses. In general, alum is one of the approved adjuvant for human use and is found in numerous vaccines e.g., HBsAg but it provoked only humoral response and poorly elicited cell mediated immunity. Recently, MF59, another adjuvant (emulsion based) was approved which is potent enhancer of both humoral and cell mediated immune responses. In 2015, Food and Drug Administration (FDA) approved a trivalent vaccine called as Fluad (formulated with MF59; effective for 65 years or more), produced from three strains of influenza (2 subtype A and 1 type B) for prevention of seasonal influenza. Now, only two adjuvants are approved i.e., alum and MF59. So, a number of scientists and researchers have focused only on selective medicinal plant products especially leaves of *C. rotang* and tried to use as adjuvant against specific protein antigen. In this study, a

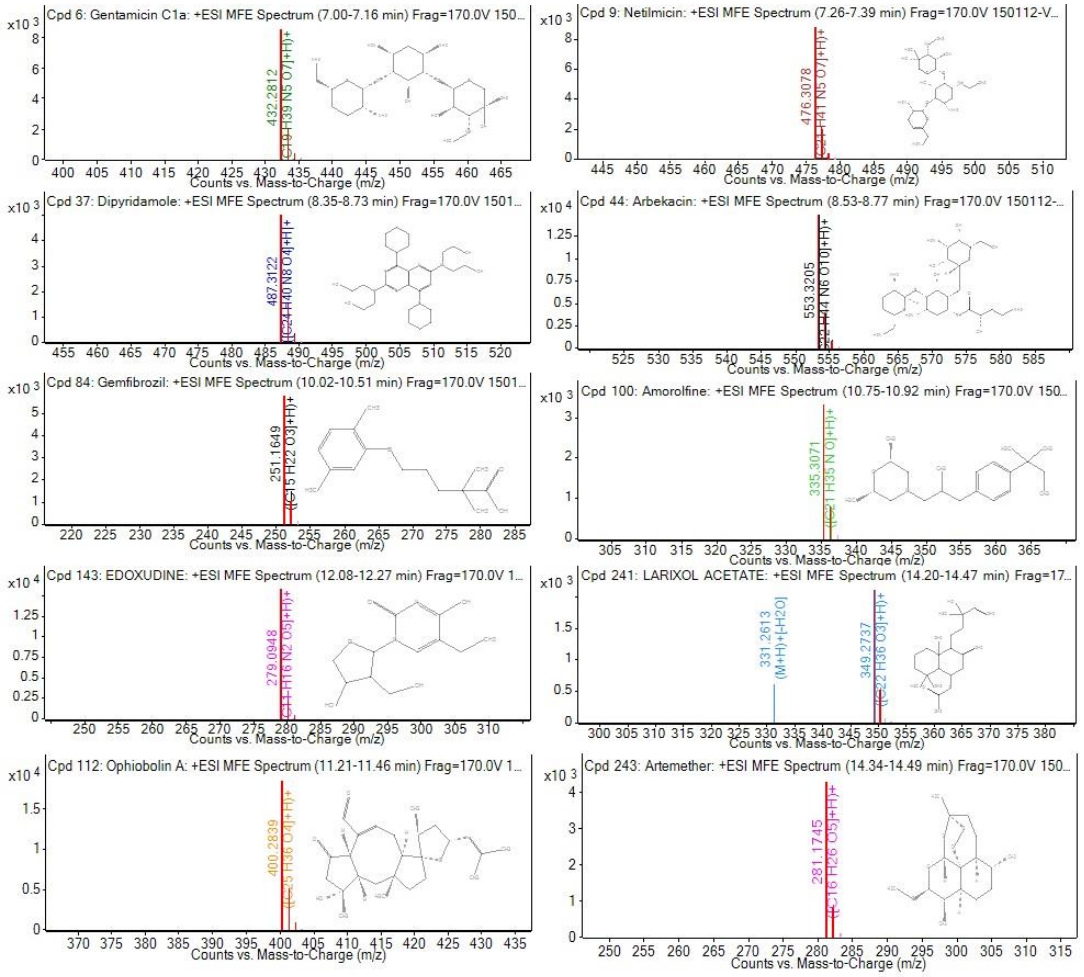

Figure 2. MS spectra for representative bioactive phytochemicals identified in *Calamus rotang*.

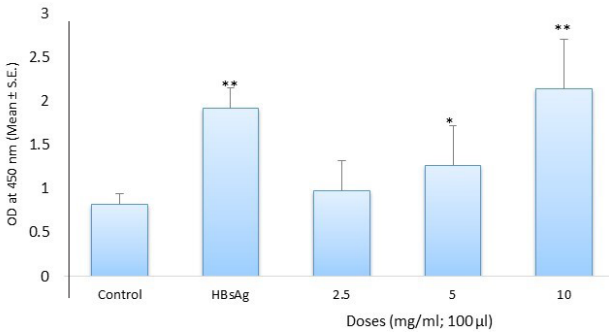

Figure 3. ELISA assay. Indirect ELISA was performed using standard HBsAg (1:1000 dilution) as coating antigen. Aqueous leaves extract were used for the estimation of anti-HBsAg antibody titre. Horse anti-serum used as secondary antibody and optical density measured at 450 nm. The difference between the control versus standard and aqueous extract were determined by one-way ANOVA test. * $P < 0.05$ and ** $P < 0.01$ versus control.

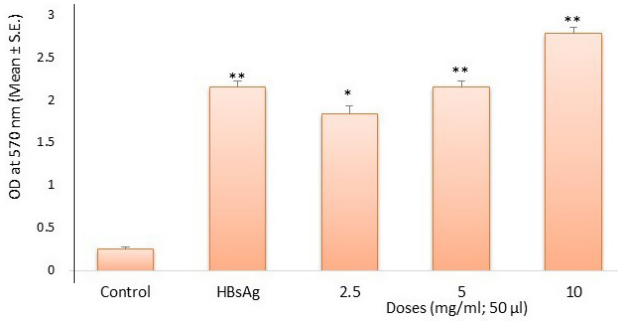

Figure 4. Effect of aqueous leaves extract of *Calamus rotang* on splenocyte proliferation assay. Spleen cells (10^6 cells/mL; 100 µL) were treated with variable doses of aqueous leaves extract in presence HBsAg (as already described in materials and methods section). Values are expressed in mean ± SE. The difference between the control versus standard and aqueous extract were determined by one-way ANOVA test. * $P < 0.05$ and ** $P < 0.01$ versus control.

number of metabolites especially secondary in the form of terpenoids, flavonoids, phenolics, saponins, glycosides etc. were reported in aqueous extract. A lot of researches have already done related to various immunopharmacological studies. Out of these secondary metabolites, saponins (e.g., QS21 and its derivatives) and glycosides (e.g., RLJ-NE-299A) are used as adjuvant for vaccines and/or are still under investigation (5,7).

For these studies, indirect ELISA was performed using variable doses of aqueous leaves extract against HBsAg and estimated whole IgG titre. The aqueous extract at higher doses showed enhancement in IgG titre as compared to control. In this experiment, color development was directly proportional to the amount of antibody (IgG) and aqueous extract at higher doses showed higher color development. Finally, this assay i.e., indirect ELISA clearly

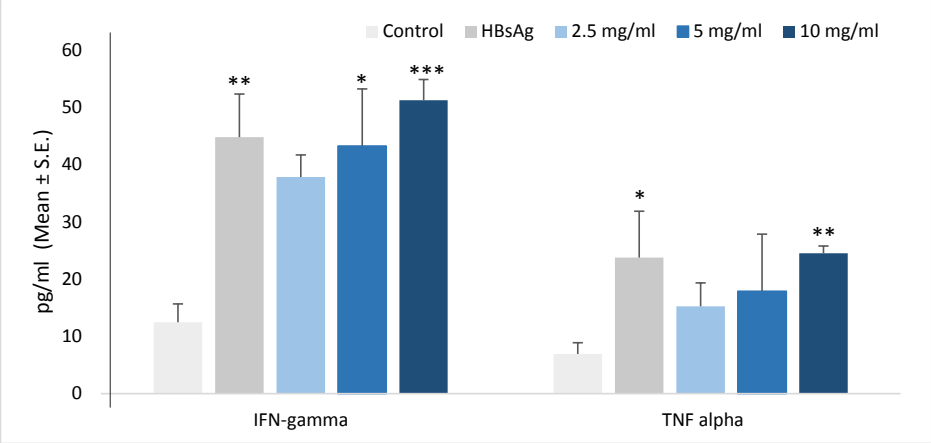

Figure 5. Effect of aqueous leaves extract of *Calamus rotang* on Th1 cytokines. Spleen cells (10^6 cells/mL; 100 µL) were treated with variable doses of aqueous leaves extract in presence HBsAg and cell culture supernatant was collected for the estimation of cytokines (IFN-gamma and TNF alpha). Values are expressed in Mean ± SE. The difference between the control versus standard and aqueous extract were determined by one-way ANOVA test. * $P < 0.05$ and ** $P < 0.01$ versus control.

indicated the enhancement in IgG titre at higher doses in aqueous leaves extract of *C. rotang* against HBsAg.

Another experiment was performed using HBsAg and determined splenocyte proliferation assay in animal model ex vivo using Swiss mice. For these studies, mice were immunized on day 0 with HBsAg and the splenocytes were collected on day 4 and determined the proliferation. The aqueous leaves extract of *C. rotang* showed enhancement in proliferation at higher doses as compared to control. In other words, HBsAg were exposed to splenic lymphocytes in presence of aqueous leaves extract. The results were dose dependent change in the proliferation as compared to HBsAg and control. Normally, HBsAg was recognized strongly by aqueous leaves extract of *C. rotang* containing primary as well as secondary metabolites and antibody-secreting cells from vaccines. Finally, there was good correlation between the aqueous leaves extract of *C. rotang* as vaccine adjuvant candidate and the conventional vaccine antigen which were tested by ELISA and proliferation assays.

Although IFN-gamma and TNF-α have been reported involved in antiviral immune responses, also they play an important role in T-cell proliferation, natural killer (NK) cell activation, and cytokine induction. Although Th1 cells secrete IFN-gamma, they activate macrophages and induce cell-mediated immunity which protect against intracellular pathogens (invasive bacteria, protozoa and viruses) (5,16). Interestingly, this study has linked the adjuvant activities of aqueous leaves extract of *C. rotang* against HBsAg. In this study, we evaluated immunoadjuvant activity of aqueous extract. It could be determined through cytokines (IFN-gamma and TNF-α) from splencoyte cell culture supernatant whether these aqueous extracts show stimulatory or suppressive effect. The aqueous extract showed enhancement in Th1 cytokines at higher doses as compared to HBsAg and control. Overall, the aqueous leaves extract of *C. rotang* showed vaccine adjuvant potential, but it is urgently needed some preservatives to store for long time in clinical trial studies.

Conclusion

The aqueous extract of *C. rotang* has immunoadjuvant activity against HBsAg and this aqueous extract has enhancement effect on IgG titre along with proliferation (splenocyte) and Th1 cytokines from cell culture supernatant. Overall, the aqueous extract of *C. rotang* also has enhancement effect on IgG titre and cell mediated immune response. Next study is recommended to emphasize on aqueous leaves extract and its safety, potency including toxicity. Also, clinical trials for the development of effective adjuvants that will help facilitate effective next-generation vaccines against devastating infectious diseases are recommended.

Authors' contributions

All the authors designed the study, wrote the protocol and interpreted the data. AG anchored the field study, gathered the initial data and performed preliminary data analysis whereas ACS for HPTLC and LC-MS analysis. All the authors managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

Conflict of interests

Authors have declared that no conflict of interest exist.

Ethical considerations

These studies were conducted under ethical guidelines with registration no. 1814/PO/ERE/S/15/CPCSEA

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