SYNERGISTIC INTERACTIONS OF PHYTOCHEMICALS IN POLYHERBAL FORMULATION ENHANCE THE CHEMICAL TRANSFORMATIONS OF ACTIVE CONSTITUENTS **

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Synergetic chemical interaction of various phytochemicals in Amruthotharam kashayam, a Polyherbal formulation, has been evaluated by different spectroscopic techniques. Comparative chemical profiling was done along with its ingredient plants by HPTLC analysis. Characterization of major chemical constituents was carried out by Fourier transform infra-red spectroscopy (FTIR) and tandem mass spectroscopic analyses (LC/MS-MS). The chromatographic profiling and spectrophotometric studies revealed that the chemistry of the finished formulation is different from that of individual plant extracts. FTIR analysis showed the spectral shift in the formulation when compared with corresponding transmittance in the ingredient plants. The synergetic chemical reaction during the process is evidenced by such specific spectral shift. The tandem mass spectroscopic studies also confirmed the chemical transformations happening during the preparation of Amruthotharam kashayam.

Keywords: *Amruthotharam kashayam, chemical transformation, Fourier transform infra-red spectroscopy, tandem mass spectroscopic analyses.*

СИНЕРГЕТИЧЕСКИЕ ВЗАИМОДЕЙСТВИЯ ФИТОХИМИКАТОВ В ТРАВЯНЫХ СМЕСЯХ, УСИЛИВАЮЩИЕ ХИМИЧЕСКИЕ ПРЕВРАЩЕНИЯ АКТИВНЫХ КОМПОНЕНТОВ

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Синергетическое химическое взаимодействие различных фитохимических веществ в многокомпонентном растительном лекарственном препарате Amruthotharam kashayam оценено различными спектроскопическими методами. Сравнительный химический анализ состава указанного препарата с параллельной оценкой вкладов отдельных растений выполнен методом высокоэффективной тонкослойной хроматографии. Определение характеристик основных химических компонентов проведено с помощью ИК-Фурье-спектрометрии (FTIR) и методов жидкостной хроматографии и тандемной масс-спектрометрии (LC/MS-MS). Хроматографическое профилирование и спектрофотометрические исследования показывают, что химический состав готовой рецептуры отличается от исходной суммы отдельных растительных экстрактов. FTIR-анализ показывает наличие спектральных сдвигов в готовом препарате по сравнению с соответствующими спектрами растений-

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ингредиентов. Указанные сдвиги свидетельствует о синергетической химической реакции в ходе образования конечного продукта. Масс-спектроскопические исследования подтверждают химические превращения в процессе приготовления Amruthotharam kashayam.

Ключевые слова: *Amruthotharam kashayam, химическое превращение, ИК-Фурье-спектрометрия, LC/MS-MS.*

Introduction. An important challenge in Ayurvedic industry is the lack of scientific veracity of its classical formulations. The holistic approach of Ayurveda emphasizes prevention of diseases, maintenance of health, and cure and management of health problems. However, it lacks evidence-based data to support the benefits of such an approach. Scientific data are absolutely essential to convince the international community about the efficacy and safety of Ayurvedic formulations. The active constituents of *Amruthotharam kashayam* (AM) have been reported earlier based on tandem mass spectroscopic analysis [1]. The therapeutic potential of an Ayurvedic formulation is due to the phytochemical constituents extracted from the ingredient plants. A single herb may even contain many phytochemical constituents, which works synergistically with each other in producing pharmacological action. Ayurvedic herbs are being used either as single drug remedies or in combinations of many. The poly-herbal formulations have the advantage of combining many plants to achieve extra therapeutic efficiency, usually known as polypharmacy or polyherbalism [2]. The ingredient plants parts of AM are *Terminalia chebula* (fruits), *Tinospora cordifolia* (stem), and *Zingiber officinale* (rhizome).

Essentially, it is the phytochemical constituents in the herbals that lead to the desired healing effect of an Ayurvedic formulation. A single herb may even contain a number of phytochemical constituents such as alkaloids, phenolics, flavonoids, tannins, saponins, terpenoids, esters, etc. that work synergistically with each other in producing pharmacological action. In the case of poly herbal preparations, the synergetic chemistry might be enhanced by several phytochemicals extracted from different ingredient plants. The aim of the present study is to evaluate the synergetic interaction of chemical constituents of *Amruthotharam kashayam* during its preparation.

Experimental. The plant materials such as *Terminalia chebula* (TCh), *Tinospora cordifolia* (TC), and *Zingiber officinale* (ZO) were collected from the Herb Garden of Arya Vaidya Sala Kottakkal and were authenticated by the Plant Systematic and Genetic Resources Division, Centre for Medicinal Plants Research (CMPR), Arya Vaidya Sala, Kottakkal, Kerala. Amruthotharam Kashayam (AM) was collected from the Product Development Department of Arya Vaidya Sala, Kottakkal, Kerala, India.

The plant materials were shade dried and chopped. Each of the samples (10 g) was successively extracted with 160 ml water using reflux extraction method for 6 h. The extracts were filtered and concentrated to 40 ml using a boiling water bath. The extracts were kept in a refrigerator until various phytochemical analyses. AM was lyophilized into powder form for various analysis.

Preliminary phytochemical screening of AM was done for the qualitative detection of major class of phytochemicals using standard procedures [3].

Total polyphenolics such as total phenolic content (TPC) and total flavonoid content (TFC) were estimated spectrophotometrically [4, 5] and were expressed as mg gallic acid equivalents (mg GaE) and mg quercetin equivalent (mg QE).

High performance thin layer chromatography (HPTLC) analysis was performed using a CAMAG HPTLC system (Switzerland). Samples were applied using CAMAG ATS-IV on aluminum backed precoated Silica gel $60F_{254}$ TLC plate (Merck India). The mobile phase was standardized as toluene, ethyl acetate, methanol, and acetic acid in a ratio of 6:3:1:0.4 for all the extracts. The chromatogram was developed in a saturated twin trough chromatographic chamber (Camag, Switzerland). The developed plate was visualized under UV at 254 and 366 nm and in visible light after derivatizing with anisaldehyde sulfuric acid reagent followed by heating at 105°C for 5 min.

The dried powdered samples of different extracts and AM were analyzed by Fourier-transform infrared spectroscopy (FTIR). The analysis was conducted on a Shimadzu-8400 FTIR system with potassium bromide (KBr) optics. The pellets were prepared in FTIR grade potassium bromide after background scan with KBr. The transmittance was measured between 400 and 4000 cm^{-1}.

Various extracts of source plants and AM were subjected to liquid chromatography/mass spectroscopic (LC/MS) analysis to identify the major chemical constituents of the AM with respect to the ingredient plants. LC-ESI/MS analysis was conducted on an Agilent 6520 accurate mass Q-TOF LC/MS system coupled with an Agilent LC 1200 system equipped with an Extend-C18 column of 1.8 μm, 2.1×50 mm. Gradient elution

was performed with LC/MS grade 0.1% acetic acid in methanol (A) and water (B) at a constant flow rate of 0.8 ml/min, with increase in the volume of B%: 5–20, 12–30, 19–40, 26–50, 30–40%. The MS analysis was performed using ESI in negative mode. The conditions for mass spectrometry were: drying gas (nitrogen) flow 5 L/min; nebulizer pressure 40 psig; drying gas temperature 325ºC; fragmentor voltage 125 V; Oct RF Vpp 750 V. The mass fragmentation was performed with varying collision energies 3V/100 DA with an offset of 6 V.

Results and discussion. Qualitative phytochemical tests were carried out to identify the major phytochemical groups present in the AM. The results are presented in Table 1. AM showed the presence of alkaloids, phenolics, flavonoids, tannins, and saponins. Phenolics and tannins are the most abundant class of compounds in this plant. This might be due to ingredient plants such as TCh and TC, which have been reported for their higher polyphenolic contents [6, 7].

TABLE 1. Preliminary Phytochemical screening of AM

Sl. No.	Test for	Present/absent
1	Alkaloids	+
2	Carbohydrates	++
3	Flavonoids	+
4	Phenolics and Tannins	+++
5	Saponins	+

Total polyphenolics such as total phenolic contents and total flavonoid contents of both plants and AM were estimated spectrophotometrically (Table 2). AM showed 261.67 mg of gallic acid equivalent phenolics per gram of the sample. Among the ingredient plants, *T. chebula* showed the highest phenolic content (192.67 mg EGa per gram), which is higher than that of previous reports [6, 8]. The difference in the TPC is due to the difference in the extraction solvent used. The previous reports were based on methanolic and aqueous methanolic extracts; however, the present study was conducted in water extract. The phenolic contents of *T. cordifoila* and *Z. officinale* are found to be much lower (8.53 and 7.6 mg EGa) when compared to that of *T. chebula*. The flavonoid content of AM is 121.67 mg EQe per gram, which is higher than that of the cumulative flavonoid content of ingredient plants. The ratio of flavonoids/phenolics (F/P) was also calculated to specify the flavonoid content in comparison with phenolics. *T. cordifolia* showed the highest F/P ratio (0.68), followed by *Z. officinale* (0.66). *T. chebula* showed the lowest F/P ratio among the ingredient plants despite its higher phenolic concentration, which indicates that most of the polyphenolics of *T. chebula* might be simple or complex phenolics other than flavonoids. It is evident that in the formulation the content of phenolics is higher when compared to the ingredient plants. In the case of poly-herbal formulation, different combinations may improve the interaction of various compounds to be extracted. The slightly acidic PH of *Z. officinale* might be a reason for the enhanced extraction of phenolics from other ingredient plants. Different components may exert a synergistic effect to enhance the effective extraction. It is very clear that AM is chemically different from that of individual ingredient plant extracts.

TABLE 2. Total Polyphenolics of *Amruthotharam Kashayam* and Ingredient Plants

Sample	TPC (mg EGa)	TFC (mg EQe)	F/P
TC	8.53±0.115	5.8±0.173	0.680
ZO	7.60±0.00	5.07±0.923	0.667
TCh	192.67±1.154	58.67±4.618	0.305
AM	261.67±2.886	121.67±5.773	0.465

The chemical profiling of AM in comparison with that of ingredient plant extracts was done by high performance thin layer chromatographic (HPTLC) analysis. HPTLC profiling of AM showed differences in chemical profile in comparison with ingredient plants. At 254 nm, AM showed major bands at 0.19, 0.47, 0.63, 0.89, and 0.97, band at Rf 0.97 was found to be common for all the ingredient plants. The bands at 0.47 and 0.89 in AM were from TCh and ZO respectively. The bands at 0.19 and 0.63 are only found in AM, which are not from any ingredient plants. A common band for both TC and TCh at 0.88 was found to be absent in AM. At 366, AM showed three bands at 0.19, 0.59, and 0.96. At 550 nm, a few new bands were ob-

served in AM at Rf 0.19, 0.56, 0.65, and 0.83. Most of the bands present in ZO were not identified in AM. HPTLC analysis showed certain new bands and disappearance of some bands that are present in the ingredient plants. The chemical profiling also confirms the synergetic chemical reaction occurring during the process of preparation of the formulation.

FTIR analysis was performed to identify the major functional groups present and to detect the spectral shift in the formulations. FTIR is a fast and nondestructive analytical method associated with chemometrics, and it is a suitable technique for analysis of herbal medicine. Moreover, it can be applied to identify the chemical changes happening during herbal drug preparation. The IR spectra of AM and individual plants showed various transmittance correspondence to the specific chemical constituents. Transmittance correspondences to compounds such as chebulic acid, gallic acid, and catechin were noticed in AM. The spectral shift between 3030 and 3100 cm^{-1} in AM may be due to certain additional small rings that are not found in ingredient plants. In the case of *Z. officinale*, the spectral shift between 1200 and 1700 cm^{-1} has been observed in the final formulations. The FTIR spectral analysis showed that most of the compounds present in the individual extract of ZO were not being exacted into the formulation. The IR spectrum of AM in comparison with that of its ingredient plants showed that there are certain chemical transformations occurring during the preparation of AM. The spectral shift also indicated the possibility of formation of certain new compounds (Fig. 1).

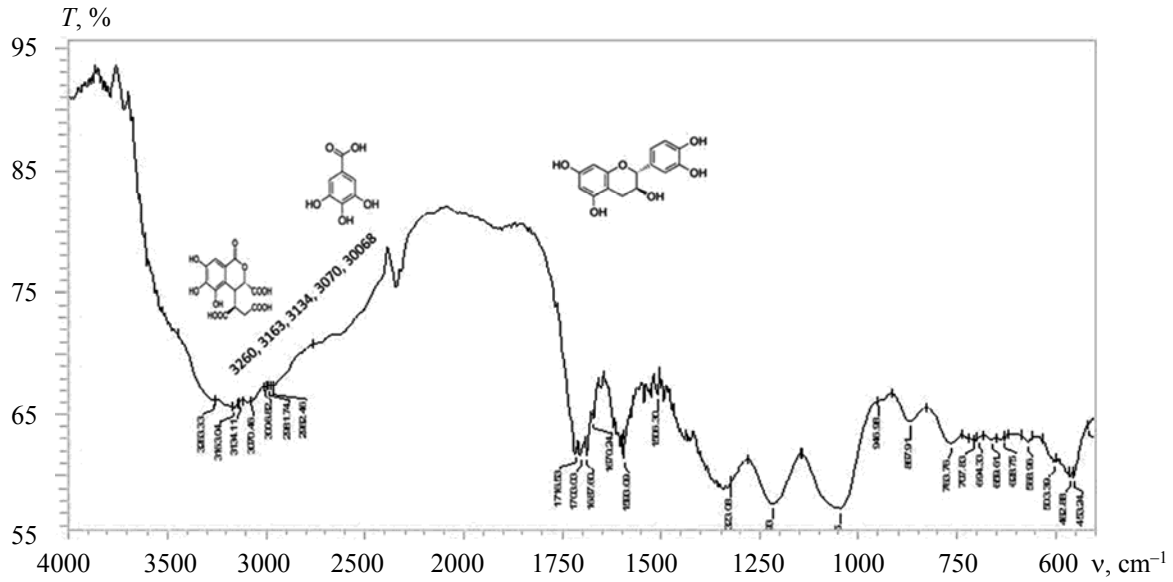

Fig. 1. FTIR spectrum of AM.

LC/MS analysis was carried out in all the ingredient plant extracts along with AM. Mass fragmentation of selected ions was done by collision induced dissociation (CID) by varying the collision energy with respect to the molecular mass (Table 3, Fig. 2). The tentative structure was identified by comparing the mass fragments with that of precursor parent ions, and the same was correlated with previous reports [9–13]. Compounds such as chebulic acid, gallic acid, catechin, shikimic acid, quinic acid, malic acid, and quercetin-3-rhamnoside were identified from AM. The ion corresponding to chebulic acid (*m*/*z* 355.023) was identified in both *T. chebula* and AM, and it might be extracted from *T. chebula* as reported earlier [6]. Gallic acid (*m*/*z* 169.015) and catechin (*m*/*z* 289.006) were also present in AM, TCh and TC. ZO also showed the presence of gallic acid. Compounds such as quinic acid and malic acid were found to be common for both AM and ingredient plants. The ZO showed the presence of many compounds such ferulic acid, sebacic acid, dihydroxyfalavanone, citramalic acid, 6-hydroxyflavone, 2-coumaric acid, etc., which were found to be absent in the AM. It was found that most of the compounds from ZO were not found in the final formulation. It is obvious that *Z. officinale* is an ingredient that affects other ingredients in the extraction medium. The therapeutic effect of herbal medicines is due to the presence of different phytoconstituents, and the effects are further potentiated when compatible herbals are formulated together. The chemical transformation resulted in formation of new compound such as quercetin-3-rhamnoside, which was not identified from any of the ingredient plants.

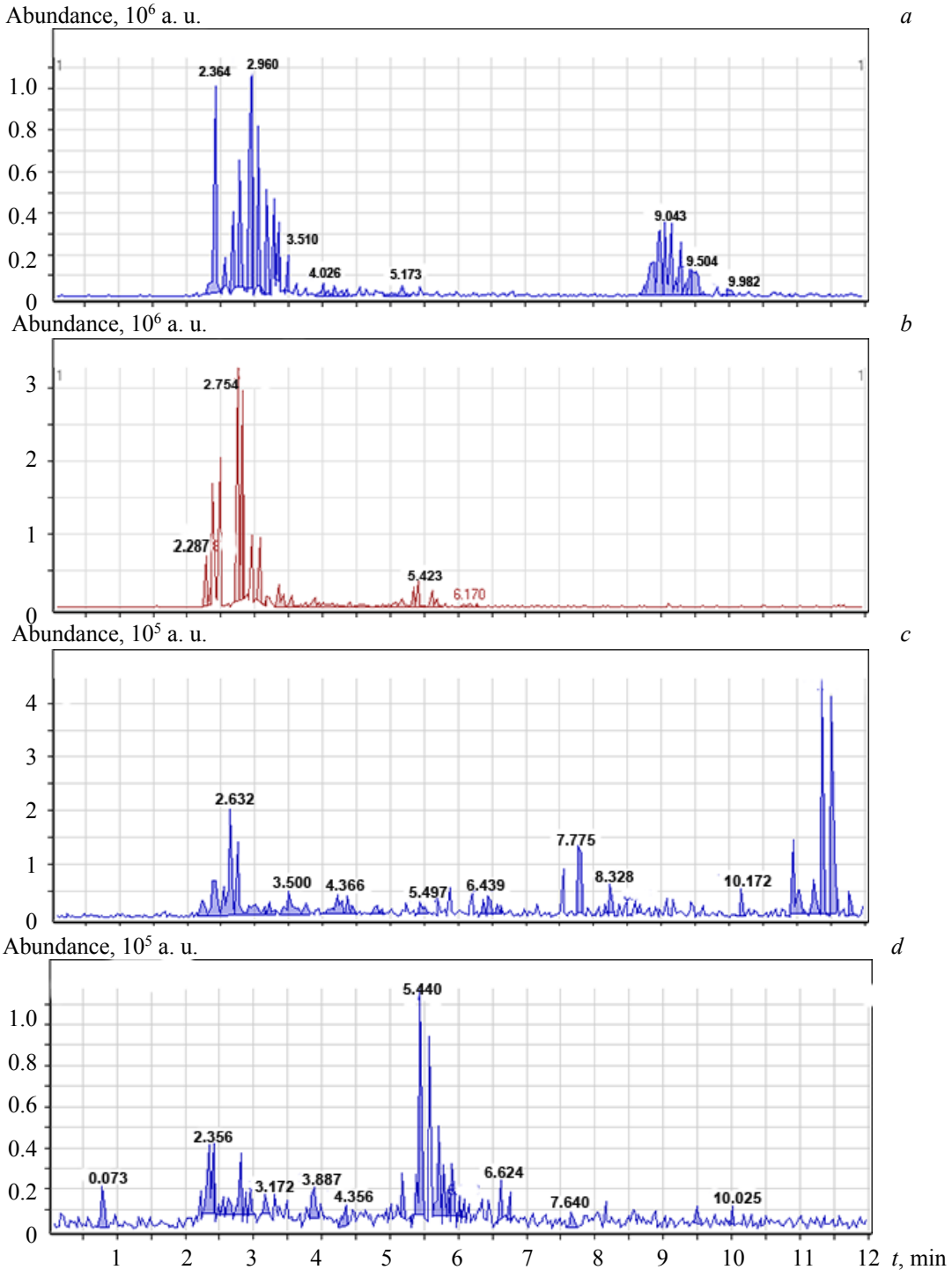

Fig. 2. LC/MS Total Ion Chromatogram of AM (a), TCh (b), TC (c), and ZO (d).

In the traditional system of Indian medicine, plant formulations and combined extracts of plants are chosen rather than individual ones. Scientific studies have revealed that plants of varying potency when combined may theoretically produce a greater result, as compared to individual use of the plant and also the sum of their individual effects. This phenomenon of positive herb-herb interaction is known as synergism. Certain pharmacological actions of active constituents of herbals are significant only when potentiated by those of other plants but are not evident when used alone [2]. The present study also establishes the synergetic interactions of chemical constituents extracted from various ingredient plants.

TABLE 3. Liquid Chromatography Mass Spectroscopy Analyses of AM and Its Ingredient Plants

Sl No.	MS (M-H)	MS/MS	Tentative Identification	Molecular formula	Identified From
1	355.023	337.02,249.05,116.95	Chebulic acid	$C_{14}H_{12}O_{11}$	AM, TCh
2	169.015	125.02	Gallic acid	$C_7H_6O_5$	AM, TCh, TC, ZO
3	289.0068	245.01	Catechin	$C_{15}H_{14}O_6$	AM, TCh, TC
4	173.0491	155.03,137.02	Shikimic acid	$C_7H_{10}O_5$	AM, TCh
5	191.0594	111.01	Quinic acid	$C_7H_{12}O_6$	AM, TCh, TC, ZO
6	133.0179	115	Malic acid	$C_4H_6O_5$	AM, TCh, TC, ZO
7	179.0777	162,135.08	Caffeic acid	$C_9H_8O_4$	TC
8	193.0913	149.1	Ferulic acid	$C_{10}H_{10}O_4$	ZO
9	447.0657	300	Quercetin -3-rhamnoside	$C_{21}H_{20}O_{11}$	AM
10	371.037	353.02,191.02	2-O-caffeoylglucaric acid	$C_{15}H_{16}O_{11}$	T.Ch
11	201.1243	183.11,139.12	Sebacic acid	$C_{10}H_{18}O_4$	ZO
12	255.245	209.12	2',6-Dihydroxyflavanone	$C_{15}H_{12}O_4$	ZO
13	115.012	114.06	Maleic acid	$C_4H_4O_4$	ZO
14	147.0751	130.95,102.06	Citramalic acid	$C_5H_8O_5$	ZO
15	130.096	112.99	5-Aminolevulinic acid	$C_5H_9NO_3$	ZO
16	237.0538	193.06	6-Hydroxyflavone	$C_{15}H_{10}O_3$	ZO
17	163.0499	119.05	2-Coumaric acid	$C_9H_8O_3$	ZO
18	174.0757	130.07,111.08	Indole-3-acetic acid	$C_{10}H_9NO_2$	ZO
19	160.0502	116.05	Indole-5-carboxylic acid	$C_9H_7NO_2$	ZO
20	295.0739	252.08, 251.08, 223.08	6-Ethoxy-3(4'-hydroxy-phenyl)4-methylcoumarin	$C_{18}H_{16}O_4$	TC
21	153.0256	109.03	2,5-Dihydroxybenzoic acid	$C_7H_6O_4$	TCh
22	477.0594	301	Quercetin-3-glucuronide	$C_{21}H_{17}O_{13}$	TCh

Conclusions. Ayurvedic formulations are gaining great importance as a cure for several health problems and are getting global attention these days. This scenario is obvious as a major increase in herbal formulation usage has been observed during the last few years in the developed world, where their market expansion has occurred in European countries and USA. The therapeutic potential of an Ayurvedic formulation is due to the phytochemical constituents extracted from the ingredient plants. The present study was conducted on an important Ayurvedic polyherbal formulation, *Amruthotharam Kashayam*, to evaluate the synergetic reaction of various phytochemicals extracted from different ingredient plants. The chromatographic profiling and spectrophotometric studies revealed that the chemical constituents of the finished formulation are different from those of individual plant extracts. The synergetic chemical reaction during the process is evidenced by the FTIR spectral shift. Tandem mass spectroscopic studies also supported the same. The present study validated the synergetic interaction of active compounds in an Ayurvedic polyherbal formulation.

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