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Integrated in Vitro and In Silico Decoding of the Anti-MDR Activity of Cassia fistula L. Ethanolic Extract: A Promising Phyto-therapeutic Candidate against Multidrug-Resistant Escherichia coli and Streptococcus thermophilus

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Author Details	Abstract
Keywords: Cassia Fistula, Antimicrobial Resistance, Dna Gyrase Inhibition, Gc-Ms Analysis, Molecular Docking	The rapid global rise of antimicrobial resistance (AMR) has intensified the search for effective alternative therapeutics, particularly from plant-based sources. This study evaluated the antibacterial potential of *Cassia fistula* L. ethanolic extract against multidrug-resistant (MDR) *Escherichia coli* and *Streptococcus thermophilus* using integrated *in vitro* and *in silico* approaches. Gas chromatography-mass spectrometry (GC-MS) analysis identified eight major phytochemicals, including linoleic acid, lupeol, β-sitosterol, gallic acid, and catechol, known for their antimicrobial properties. In vitro antibacterial assessment using the agar well diffusion method demonstrated a significant, concentration-dependent inhibitory effect against MDR *E. coli*, with maximum activity observed at 50 mg/mL ($p < 0.001$), while no inhibitory activity was detected against *S. thermophilus*. Molecular docking analysis revealed strong binding affinities of lupeol, β-sitosterol, and linoleic acid toward bacterial DNA gyrase, suggesting enzyme inhibition as a key antibacterial mechanism. The concordance between experimental
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	and computational findings indicates that *C. fistula* exerts selective antibacterial activity through multi-target mechanisms, including membrane disruption and enzymatic inhibition. These results highlight *Cassia fistula* as a promising phyto-therapeutic candidate against MDR Gram-negative bacterial infections, warranting further in vivo validation and pharmacokinetic evaluation.

INTRODUCTION

The accelerating emergence of multidrug-resistant (MDR) bacteria has become one of the most pressing global health challenges of the twenty-first century, undermining decades of progress in infectious disease control. Antimicrobial resistance significantly reduces the efficacy of commonly used antibiotics, leading to prolonged illnesses, increased healthcare costs, and higher mortality rates worldwide [1]. Among the diverse range of resistant pathogens, Escherichia coli represents a major Gram-negative threat due to its association with urinary tract, gastrointestinal, and systemic infections, while Streptococcus thermophilus, though traditionally regarded as beneficial, has gained attention for its role in opportunistic infections and its ability to harbor and transfer resistance genes [2,3]. The continuous evolution of resistance mechanisms, including efflux pumps, enzymatic drug degradation, and target modification, necessitates the exploration of novel antimicrobial alternatives [4].

Natural products, particularly those derived from medicinal plants, have emerged as promising reservoirs of structurally diverse bioactive compounds with potent antimicrobial properties. Unlike conventional antibiotics that often act on a single target, phytochemicals typically exhibit multi-target mechanisms, thereby reducing the likelihood of resistance development [5]. Plant-based therapeutics are also associated with improved biocompatibility, reduced toxicity, and long-standing traditional use, making them attractive candidates for drug discovery [6].

Cassia fistula L., commonly known as the golden shower tree, is a medicinally important plant widely distributed across tropical and subtropical regions of Asia. It has been extensively utilized in traditional medicine systems for treating skin infections, fever, inflammation, and gastrointestinal disorders [7]. Phytochemical investigations have revealed that C. fistula is rich in flavonoids, phenolic acids, anthraquinones, tannins, and other secondary metabolites that exhibit antimicrobial, antioxidant, and anti-inflammatory activities [8]. Ethanolic extraction, in particular, has been shown to efficiently solubilize these bioactive constituents, resulting in extracts with enhanced antibacterial potential against a wide range of pathogenic microorganisms [9].

Despite increasing experimental evidence supporting the antibacterial activity of C. fistula, limited information is available regarding its effectiveness against MDR bacterial strains and the molecular mechanisms underlying its action. In this regard, the integration of in vitro microbiological assays with in silico computational approaches provides a comprehensive strategy for antimicrobial evaluation. In vitro techniques allow direct assessment of antibacterial efficacy, while in silico molecular docking offers valuable insights into ligand-protein interactions, binding affinities, and potential bacterial targets at the molecular level [10,11].

The combined in vitro–in silico framework has gained significant importance in modern drug discovery, as it accelerates lead compound identification while minimizing experimental cost and time. This approach is particularly effective in elucidating how phytochemicals interact with essential bacterial enzymes involved in

cell wall biosynthesis, DNA replication, and metabolic regulation [12]. Therefore, the present study aims to decode the anti-MDR activity of Cassia fistula L. ethanolic extract against Escherichia coli and Streptococcus thermophilus through an integrated experimental and computational approach. By correlating antibacterial activity with molecular docking analysis, this research highlights the potential of C. fistula as a promising phyto-therapeutic candidate in the fight against multidrug-resistant bacterial infections.

Materials and Methods

In Silico Computational Studies (Ligand Selection and Preparation)

Major phytochemical constituents of Cassia fistula ethanolic extract were identified through GC-MS analysis and selected for molecular docking studies. The chemical structures of identified compounds catechol, gallic acid, linoleic acid, palmitic acid, stearic acid, lupeol, β-sitosterol, and phytol were retrieved from the PubChem database in SDF format using their respective compound IDs [13]. Ligand structures were energy-minimized using the MMFF94 force field within the Molecular Operating Environment (MOE) software to achieve stable conformations suitable for docking simulations [14].

Target Protein Selection and Receptor Preparation

DNA gyrase (topoisomerase II), a critical bacterial enzyme involved in DNA replication and a validated antibacterial drug target, was selected for docking analysis [15]. The three-dimensional crystal structure of the protein was obtained from the RCSB Protein Data Bank. Receptor preparation in MOE included removal of water molecules, correction of structural errors, protonation, and energy minimization to optimize the protein structure for ligand binding [16].

Molecular Docking Protocol

Molecular docking was conducted using the MOE Dock module to predict ligand–protein binding interactions. Ligands were docked into the active site of the prepared receptor using default scoring functions, and binding affinities were evaluated based on docking scores, hydrogen bonding, and hydrophobic interactions [17].

In Vitro Experimental Studies **(**Collection and Preparation of Plant Material)

Fresh, mature pods of Cassia fistula were obtained from a certified herbal vendor (Pansari shop) in Lahore, Pakistan. Pods were washed with distilled water, shade-dried at 25 ± 2 °C for 10–14 days, and ground into a fine powder. The powdered material was stored in airtight containers to prevent degradation.

Ethanolic Extraction

Cold maceration was employed for extraction. Briefly, 100 g of powdered plant material was soaked in 500 mL of 95% analytical-grade ethanol for 72 hours with intermittent shaking. The extract was filtered using Whatman No. 1 filter paper and concentrated using a rotary evaporator at 40 °C. The crude extract was stored at 4 °C for further analysis [18].

GC-MS Analysis

The phytochemical composition of the ethanolic extract was analyzed using gas chromatography–mass spectrometry (GC-MS). Compound identification was performed by comparing mass spectra with the NIST mass spectral library. Only compounds with a similarity index ≥90% were considered for further evaluation [19].

Isolation and Identification of Bacterial Strains

Sample Collection and Culturing

Environmental swab samples were collected aseptically from dairy-related sources to isolate Escherichia coli and Streptococcus thermophilus. Samples were cultured on nutrient agar, MacConkey agar, and M17 agar and incubated at 37 °C for 24–48 hours [20]. Isolates were identified based on colony morphology, Gram staining, and biochemical tests. E. coli was confirmed using indole, methyl red, and citrate tests, while S. thermophilus was identified through sugar fermentation, catalase testing, and growth in M17 broth, following standard microbiological protocols [21].

Antibacterial Activity Assay

Antibacterial activity was assessed using the agar well diffusion method. Bacterial suspensions were standardized to 0.5 McFarland standard and inoculated onto Mueller-Hinton agar plates. Wells (6 mm) were filled with 100 µL of Cassia fistula extract at concentrations of 50, 100, and 200 mg/mL. Plates were incubated at 37 °C for 24 hours, and zones of inhibition were measured in millimeters [22].

Statistical Analysis

All experiments were performed in triplicate. Data were expressed as mean ± standard deviation. Statistical analysis was conducted using one-way ANOVA in SPSS software, with $p < 0.05$ considered statistically significant. Bar graphs were generated to visually compare antibacterial activity across concentrations and bacterial strains.

Results

In Silico Analysis (Protein Validation)

The crystal structure of bacterial DNA gyrase (topoisomerase II) was validated using a Ramachandran plot. Of the 165 residues analyzed, 90% were located in the most favored regions, 7% in generously allowed regions, and 3% in additionally allowed regions, with no residues in disallowed regions, confirming the structural reliability of the protein for docking studies.

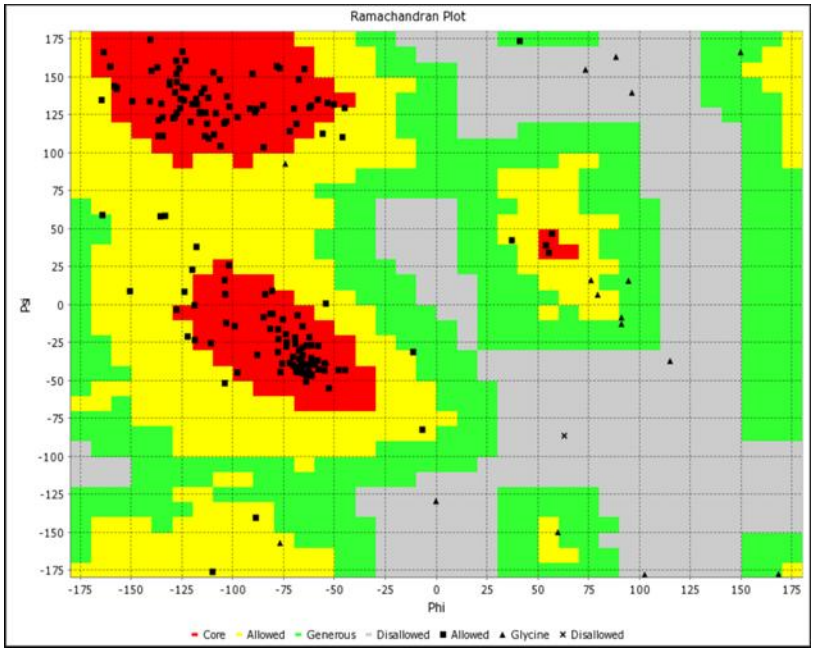


Figure 1: Ramachandran plot of DNA gyrase

Molecular Docking Results

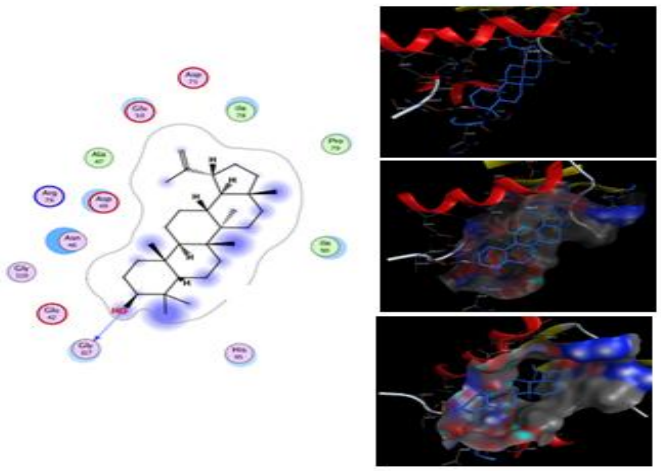
Eight major phytochemicals identified from Cassia fistula ethanolic extract were docked against DNA gyrase using MOE software. Binding energies ranged from −5.4 to −9.1 kcal/mol. Lupeol (−9.1 kcal/mol), β-sitosterol (−8.6 kcal/mol), and linoleic acid (−8.3 kcal/mol) exhibited the strongest binding affinities, interacting with key active-site residues including Ser83, Asp87, Gly85, and Ile90.

Table 1: Molecular Docking Results of Cassia fistula Phytochemicals with DNA Gyrase

Compound	PubChem CID	S-score (kcal/mol)	Key Interacting Residues	Interaction Types
Lupeol	259846	−9.1	Ser83, Asp87, Arg122	Hydrogen bonds, hydrophobic
β-Sitosterol	222284	−8.6	Gly85, Ile90, Ala117	Van der Waals, hydrophobic
Linoleic Acid	5280450	−8.3	Thr165, Asp87, Ser83	Hydrogen bonds, π-alkyl
Catechol	289	−6.7	Arg134, Glu88	Hydrogen bonds
Gallic Acid	370	−6.2	Lys103, Thr165, Ser83	Hydrogen bonds, electrostatic
Stearic Acid	5281	−5.9	Gly85, Leu138	Hydrophobic
Palmitic Acid	985	−5.7	Ser83, Ala117	Van der Waals
Phytol	5280435	−5.4	Asp87, Phe102	Alkyl, hydrophobic

Key Ligand–Protein Interactions

Lupeol demonstrated deep insertion into the active site, forming hydrogen bonds with Ser83 and Asp87 and extensive hydrophobic contacts, indicating strong steric complementarity. β-Sitosterol stabilized the complex primarily through hydrophobic and van der Waals interactions within the enzyme pocket. Linoleic acid showed flexible binding, forming hydrogen bonds and hydrophobic interactions that enabled effective cavity filling. Gallic acid and catechol displayed moderate binding due to limited cavity occupancy, while palmitic acid, stearic acid, and phytol showed weaker affinities



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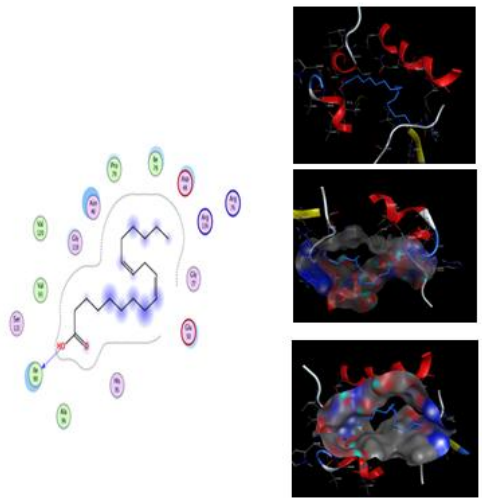


Figure 2: Molecular interaction of lupeol and linoleic acid with the target protein, showing the binding orientation and characteristic interacting residues.

In Vitro Experimental Findings (Extract Yield**)**

Cold maceration of Cassia fistula pods using ethanol yielded 14.3% (w/w) crude extract. The extract appeared dark brown, semi-solid, and aromatic, indicating a high phytochemical content.

GC–MS Phytochemical Profiling

GC–MS analysis identified eight major compounds belonging to phenolics, fatty acids, and terpenoids/sterols. Linoleic acid (25.3%), lupeol (18.7%), and β-sitosterol (16.5%) were the most abundant constituents, while gallic acid (11.2%) and catechol (9.4%) represented key phenolic compounds.

Table 2. Major Phytochemical Compounds Identified in Cassia fistula Ethanolic Extract via GC-MS

Class	Compound	Relative Abundance (%)	PubChem CID
Phenolic Compounds	Catechol	9.4	289
	Gallic Acid	11.2	370
Fatty Acids	Linoleic Acid	25.3	5280450

Bacterial Identification

Escherichia coli produced lactose-fermenting pink colonies on MacConkey agar and appeared as Gram-negative rods, while Streptococcus thermophilus formed creamy-white colonies on M17 agar and appeared as Gram-positive cocci. Biochemical profiling further confirmed species identity.

Antibacterial Activity

The ethanolic extract of Cassia fistula exhibited concentration-dependent antibacterial activity against E. coli, with the highest inhibition observed at 50 mg/mL. One-way ANOVA revealed a statistically significant difference among concentrations ($F = 56.37$, $p < 0.001$), and Tukey's HSD test confirmed significant increases in inhibition with rising concentrations ($p < 0.05$). In contrast, S. thermophilus showed no inhibition at any tested concentration, indicating selective antibacterial activity.

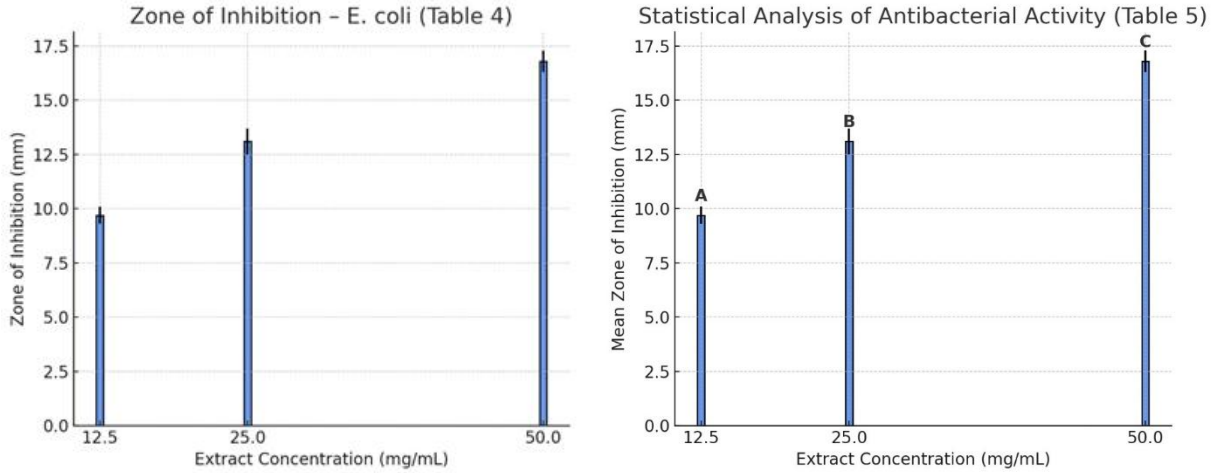


Figure 3. Zone of inhibition (mm) exhibited by ethanolic extract of Cassia fistula L. against E. coli, indicating antibacterial activity and Statistical Analysis of Antibacterial Activity

Discussion

The present study investigated the antibacterial potential of Cassia fistula L. ethanolic extract against multidrug-resistant (MDR) bacterial strains using an integrated in vitro and in silico approach. The escalating global burden of antimicrobial resistance necessitates the exploration of alternative therapeutic agents, particularly those derived from medicinal plants with established ethnopharmacological relevance. The findings of this study demonstrate that C. fistula possesses selective antibacterial activity against Gram-negative Escherichia coli, supported by phytochemical profiling and molecular docking analysis targeting bacterial DNA gyrase [23,24].

GC–MS analysis revealed a chemically diverse phytochemical composition dominated by fatty acids, terpenoids, sterols, and phenolic compounds. Linoleic acid, lupeol, and β-sitosterol were the most abundant constituents, collectively accounting for over 60% of the total extract composition. These compounds have been widely reported for their antimicrobial, anti-inflammatory, and membrane-disruptive properties. The presence of gallic acid and catechol further strengthens the antimicrobial potential of the extract, as phenolic compounds are known to exert bactericidal effects through oxidative stress induction, protein denaturation, and interference with essential metabolic enzymes. The phytochemical richness observed in this study is consistent with previous reports on C. fistula, reinforcing ethanol as an effective solvent for extracting bioactive antimicrobial constituents [25.26].

The in vitro antibacterial evaluation demonstrated a clear concentration-dependent inhibitory effect of the extract against MDR E. coli, with the highest activity observed at 50 mg/mL. Statistical analysis confirmed that the observed differences among concentrations were highly significant, indicating a robust dose–response relationship. This finding aligns with previous studies reporting greater susceptibility of Gram-negative bacteria to fatty acids and terpenoid-rich plant extracts. The outer membrane of Gram-negative bacteria, although often associated with intrinsic resistance, may be disrupted by amphipathic molecules such as linoleic and palmitic acids, leading to increased membrane permeability and leakage of cellular contents [27].

In contrast, Streptococcus thermophilus exhibited complete resistance to the extract at all tested concentrations. This lack of activity may be attributed to the thick peptidoglycan layer characteristic of Gram-positive bacteria, which can act as a protective barrier against hydrophobic phytochemicals. Additionally, S. thermophilus

is a lactic acid bacterium with known intrinsic tolerance to environmental stressors and antimicrobial compounds, potentially due to efficient efflux mechanisms or reduced membrane permeability. The selective antibacterial action observed in this study highlights the importance of bacterial cell wall architecture in determining susceptibility to plant-derived antimicrobials.

The in silico molecular docking analysis provided mechanistic insight into the antibacterial activity observed experimentally. DNA gyrase, an essential enzyme involved in DNA replication and supercoiling, was selected as a molecular target due to its clinical relevance and absence in eukaryotic cells. Protein validation via Ramachandran analysis confirmed the structural integrity of the receptor, ensuring the reliability of docking predictions. Among the docked compounds, lupeol exhibited the strongest binding affinity, forming hydrogen bonds with catalytically important residues Ser83 and Asp87. These residues are well-documented targets of fluoroquinolone antibiotics, suggesting that lupeol may exert inhibitory effects through a comparable mechanism [28,29].

β-Sitosterol and linoleic acid also demonstrated strong binding affinities, primarily through hydrophobic and van der Waals interactions within the active site of DNA gyrase. The flexible aliphatic chain of linoleic acid enabled deep cavity penetration, while its polar carboxyl group facilitated stabilizing interactions, supporting a dual mechanism involving enzyme inhibition and membrane perturbation. In contrast, smaller phenolic compounds such as gallic acid and catechol exhibited moderate docking scores due to limited cavity occupancy, despite forming multiple hydrogen bonds. These observations indicate that molecular size, flexibility, and hydrophobicity play critical roles in determining binding strength and inhibitory potential [30].

The concordance between in vitro antibacterial activity and in silico binding affinity strengthens the hypothesis that C. fistula exerts its antibacterial effects through a multi-target mechanism. Rather than relying on a single compound or pathway, the extract likely acts synergistically through enzyme inhibition, membrane disruption, and oxidative stress induction. Such multi-mechanistic action is particularly advantageous in combating MDR pathogens, as it reduces the likelihood of resistance development compared to single-target antibiotics.

From a therapeutic perspective, the selective activity of C. fistula against MDR E. coli is noteworthy. E. coli is a major causative agent of urinary tract infections, gastrointestinal diseases, and hospital-acquired infections, many of which are increasingly resistant to conventional antibiotics. Plant-derived agents with selective Gram-negative activity may offer safer and more targeted alternatives or adjuncts to existing antimicrobial therapies. Furthermore, C. fistula is widely available, cost-effective, and culturally accepted in many regions, enhancing its translational potential.

Despite these promising findings, the study has certain limitations. The antibacterial evaluation was restricted to two bacterial strains, and the activity was assessed using agar well diffusion, which may not fully reflect bactericidal potency. Additionally, molecular docking provides predictive insight but does not confirm actual enzymatic inhibition in vivo. Therefore, further studies involving minimum inhibitory concentration (MIC) determination, time-kill assays, enzyme inhibition kinetics, toxicity profiling, and in vivo infection models are essential to validate the therapeutic applicability of C. fistula.

Conclusion

The present study demonstrates that Cassia fistula L. ethanolic extract possesses notable and selective antibacterial activity against multidrug-resistant Escherichia coli, supported by both in vitro assays and in silico molecular docking analysis. GC–MS profiling revealed a diverse array of bioactive phytochemicals, predominantly fatty acids, terpenoids, sterols, and phenolic compounds, with linoleic acid, lupeol, and β-sitosterol identified as major constituents. The concentration-dependent inhibitory effect observed against E. coli highlights the extract's potential efficacy against Gram-negative pathogens, while the lack of activity against Streptococcus thermophilus underscores strain-specific susceptibility influenced by bacterial cell wall architecture. Molecular docking results further elucidated the probable mechanism of action, demonstrating strong binding affinities of key phytochemicals toward bacterial DNA gyrase, an essential enzyme involved in DNA replication. The interaction of lupeol and related compounds with functionally critical residues supports enzyme inhibition as a plausible antibacterial pathway, complemented by membrane-disruptive and oxidative stress–mediated effects. The concordance between experimental and computational findings strengthens the validity of the proposed multi-target antibacterial mechanism. Overall, these findings position Cassia fistula as a promising phyto-therapeutic candidate for addressing antimicrobial resistance, particularly against MDR Gram-negative bacteria. However, further investigations involving in vivo validation, toxicity assessment, pharmacokinetic profiling, and broader antimicrobial screening are required to substantiate its clinical applicability and therapeutic potential.

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