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Compatibility study of *Trichoderma* sp. with Chemical Fungicides Commonly Used by Nepalese Farmers, Under *In-Vitro* Condition

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Abstract

The excessive use of synthetic fungicides has led to the emergence of fungicide-resistant strains of pathogens, raising concerns about human health and environmental impact. *Trichoderma* spp., an endophytic and versatile opportunistic plant symbiont, has recently gained popularity as a biocontrol agent. Integrated use of *Trichoderma* with compatible fungicides gives better disease management and causes less harm to the environment in the long run. This study aimed to evaluate the compatibility of six fungicides commonly used by Nepalese farmers at concentrations of 250, 500, and 1000 ppm with *Trichoderma* sp. using the poisoned food technique in-vitro. The experiment was conducted in a completely randomized design with four replications for each treatment, and data were taken at 24, 36, 48, 60, and 72 hours after incubation. Among the fungicides tested, Mancozeb was compatible at all concentrations from the start and exhibited the highest compatibility at 72 hours, with a growth inhibition percent of 0.39%. Metalaxyl+Mancozeb at 250 and 500 ppm, and Copper oxychloride at 250 ppm showed good compatibility. However, Carbendazim, Hexaconazole, and Carbendazim+Mancozeb resulted in 100% growth inhibition of *Trichoderma* sp. throughout the experiment. While all treatments significantly reduced mycelial diameter, Mancozeb and Metalaxyl+Mancozeb showed a gradual decrease in growth inhibition percent over time, indicating increased compatibility. In contrast, Copper oxychloride demonstrated variable growth inhibition percent over time. So, the fungicides Mancozeb, Metalaxyl+Mancozeb and Copper oxychloride at compatible concentrations can be used with *Trichoderma* sp. in integrated disease management to control soil and seed-borne pathogens.

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Introduction

The Green Revolution, a transformative milestone in agricultural modernization, led to a fourfold increase in fertilizer use, a sevenfold increase in nitrogen fertilizer consumption, and the widespread adoption of various agrochemicals. These changes resulted in a remarkable 145% increase in global food production and 5% more food per capita (Pretty and Bharucha, 2014). Since the beginning of the Green Revolution, chemical fungicides have become synonymous with increased production and improved disease management. While the use of these agricultural inputs has significantly boosted crop productivity and quality over the years, their excessive and improper use has led to environmental pollution and detrimental effects. These harmful practices not only harm soil beneficial microbes but also weaken the natural antagonistic activity (Meena et al., 2020).

In this scenario, it is essential to seek eco-friendly microbial alternatives to combat plant diseases. *Trichoderma* spp., widely recognized as biocontrol agents, are free-living fungi commonly found in soil and root ecosystems. These fungi can induce both localized and systemic resistance in various plants against a range of pathogens, significantly influencing plant growth, development, and immunity in both field and greenhouse conditions (Salas-marina et al., 2015; Saravanakumar et al., 2016). *Trichoderma* can be applied for seed treatment, seed biopriming, seedling treatment, soil applications, and foliar applications (Benitez et al., 2004). The use of *Trichoderma* spp. under *in-vivo* conditions has shown positive effects on plant growth parameters such as height, collar diameter, number of leaves, root size, leaf area, as well as dry mass of both roots and aerial parts (Campos et al., 2020).

Trichoderma spp. stands out as a highly utilized fungal biocontrol agent employed for managing various phytopathogens, commercially marketed as a biofertilizer, biopesticide, and for bioremediation purposes (Kumar et al., 2014; Singh et al., 2013). Among its diverse species, T. *harzianum*, T. *atroviride*, T. *asperellum*, T. *polysporum*, and T. *viride* are prominently favored biocontrol agents (Srivastava et al., 2015). Like rhizobia and mycorrhiza, *Trichoderma* does not form strict symbiotic associations with plants, but it has evolved multiple mechanisms, such as competition, antibiosis, mycoparasitism, induction of resistance, and endophytic activity, to attack other fungi and plays a crucial role in the control of seed and soil-borne diseases (Mukherjee et al., 2013) viz. *Pythium aphanidermatum* (Kipnegeno 2015), *Phytophthora infestans* (Kerroum et al., 2015), *Rhizoctonia solani* (Poudel et al., 2023), *Sclerotium rolfsii* (Poudel et al., 2023; Pacheco et al., 2016), *Fusarium sudanense* (Larran et al., 2020) etc. *Trichoderma* produces secondary metabolites that are harmful to a diverse array of soil-borne pathogens.

Furthermore, certain species of *Trichoderma* secrete a variety of hydrolytic enzymes such as cellulases, chitinases, glucanases, proteases and xylanases, which break down the cell walls of pathogenic fungi (Sood et al., 2020). As a biological organism, *Trichoderma*'s effectiveness in biocontrol can be influenced by factors like its shelf-life, soil pH, ambient temperatures, salinity, moisture levels, competition, and disease prevalence (Naeimi et al., 2020, Mukhrejee et al., 2013). Different biotic and abiotic stresses harm its biocontrol potential, so the application of only *Trichoderma* may give an inconsistent performance and low level of disease control. Korsten and Jeffries (2000) found that biocontrol agents can be more effective when used in combination with recommended fungicides at lower concentrations, as evidenced by previous studies showing improved disease management with *Trichoderma*-chemical combinations (Mahesh et al., 2010, Animisha and Zacharia 2011). However, certain chemicals negatively impact the growth and establishment of *Trichoderma* (Sushir et al., 2015). In Nepal, data on the use of chemical pesticides in agricultural crops

indicates widespread and indiscriminate application, particularly in vegetable cultivation (Bhandari et al., 2018, Gyawali 2018). This contributes to the emergence of pathogen strains resistant to fungicides, creating demand for more poisonous chemicals, so it is of utmost necessity to screen out safer chemicals and their sub-lethal concentration that causes no harm to *Trichoderma*. And, the chemicals used in this experiment are the most obvious chemicals used by Nepalese farmers, and to date, there are only a few works conducted on the compatibility of these chemicals with *Trichoderma*, so much information is yet to be documented.

Materials and Methods

Six different chemical fungicides, listed in Table 1, were assessed at three varying concentrations (250 ppm, 500 ppm, and 1000 ppm) to study their compatibility with *Trichoderma* sp. using the food poisoned technique, following the method described by Nene and Thapliyal (1993). Pure culture of *Trichoderma* sp. was procured from Nepal Plant Disease and Agro Associates (NPDA).

Table 1. Chemical fungicides used in a study

S.N.	Trade Name	Active Ingredient	Mode of Action
1	Uthane M-45	Mancozeb 75%WP	Contact
2	Blutoxx	Copper Oxychloride 50%WP	Contact
3	Saaf	Carbendazim 12% + Mancozeb 63% WP	Systemic + Contact
4	Kriloxyl Gold	Metalaxyl 8% + Mancozeb 64% WP	Systemic + Contact
5	Navistin	Carbendazim 50% WP	Systemic
6	Hexa	Hexaconazole 5% EC	Systemic

Stock solutions of fungicides were made by mixing 1 gram of each with 10 ml of sterile distilled water. The calculated volume of respective stock solution was then added in lukewarm molten PDA and mixed thoroughly by shaking the flask to prepare desired concentrations of poisoned media. The media were then poured into sterilized 90 mm Petri-plates. PDA media without fungicides served as control. After solidification, the plates were inoculated with 5 mm mycelial discs taken from the periphery of 3 days old *Trichoderma* sp. Four replications were maintained for each treatment and inoculated petri plates were incubated at 25±2°C in a bacteriological incubator. After 24 hrs, 36 hrs, 48 hrs, 60 hrs, and 72 hrs of incubation, mycelial growth diameter of *Trichoderma* sp. was recorded. Then, the growth inhibition percent was calculated by using Vincent (1947) formula.

$$\text{Growth Inhibition Percent (GIP)} = [C\text{-}T/C] \times 100$$

Where : C = Growth in unamended medium
T= Growth in amended treatment

Statistical Analysis

The data underwent analysis of variance (ANOVA) using a completely randomized design (CRD) in R (version 3.5.3). Least significant difference (LSD) was calculated at 1% level to determine the significant difference between the treatments.

Results and Discussion

Results

As shown in Figure 1 and Table 2 and 3, all the tested fungicides at all the concentrations exerted a varying degree of inhibition in the radial mycelial growth of *Trichoderma* sp as compared to control. Among the fungicides tested, Metalaxyl+Mancozeb at concentrations of 250 ppm and 500 ppm, as well as Copper oxychloride at 250 ppm, exhibited lower GIP. Mancozeb demonstrated compatibility with *Trichoderma* sp., revealing relatively low GIP at both lower (250 ppm) and higher concentrations (500 ppm and 1000 ppm) from the onset of the experiment.

Here, at 24 hrs of incubation, the data presented in Table 2 and 3 lucidly revealed that Copper oxychloride was the most compatible fungicide with mycelium growth of 2.35 and GIP of 29.22% which was statistically at par ($P<0.01$) with 250 ppm of Mancozeb and Meatalaxyl+Mancozeb showing GIP of 32.22% and 34.24%, respectively. After 36 hrs of incubation, the highest compatibility was found on Mancozeb 250 ppm with mycelium growth of 3.83 cm and GIP of 27.40% which was statistically at par ($P<0.01$) with Copper oxychloride 250 ppm with GIP of 28.98%, followed by Mancozeb 500 ppm and Metalaxyl+Mancozeb 250 ppm.

Similarly, after 48 hrs of incubation, Mancozeb 250 ppm showed the highest compatibility with mycelium diameter of 5.88 cm and GIP of 19.07% which was statistically at par ($P<0.01$) with its 500 ppm showing GIP of 23.20% and 250ppm of Metalaxyl+Mancozeb with GIP of 19.99%. After 60 hrs of incubation, the highest compatibility was noted on Mancozeb 250 ppm with a mycelium diameter of 7.52 cm and GIP of 8.89% which was followed by its 500 ppm concentration with mycelium diameter of 7.1 cm and GIP of 13.94%. Furthermore, at 72 hrs of incubation, still, Mancozeb 250 ppm showed the highest compatibility with a mycelium diameter of 8.47cm and GIP of 0.39% which was statistically at par ($P<0.01$) with its 500 ppm concentration with a mycelium diameter of 8.47cm and GIP of 0.59%. Among the fungicides tested, the biocontrol agent was sensitive to all the tested concentrations of Carbendazim, Carbendazim+Mancozeb, and Hexaconazole with a constant GIP of 100% throughout the experiment, and proved utterly incompatible with lethal effect on the *Trichoderma* sp, and hence aren't suitable in integration with *Trichoderma* sp in integrated disease management.

Table 2. Mycelial growth diameter of *Trichoderma* sp in media amended with different chemical fungicides at various concentrations under *in vitro* condition

Treatments	Conc (ppm)	Mycelial diameter (cm)				
		24 hrs	36 hrs	48 hrs	60 hrs	72 hrs
Control		3.32	5.28	7.27	8.25	8.5
Mancozeb	250	2.25	3.83	5.88	7.52	8.47
	500	2.07	3.53	5.58	7.1	8.45
	1000	1.85	3.27	5.17	6.50	8.00
Carbendazim	250	0.00	0.00	0.00	0.00	0.00

	500	0.00	0.00	0.00	0.00	0.00
	1000	0.00	0.00	0.00	0.00	0.00
Hexaconazole	250	0.00	0.00	0.00	0.00	0.00
	500	0.00	0.00	0.00	0.00	0.00
	1000	0.00	0.00	0.00	0.00	0.00
Carbendazim	250	0.00	0.00	0.00	0.00	0.00
+ Mancozeb	500	0.00	0.00	0.00	0.00	0.00
	1000	0.00	0.00	0.00	0.00	0.00
Metalaxyl +	250	2.18	3.50	5.82	6.82	8.00
Mancozeb	500	1.63	3.10	4.97	5.80	6.95
	1000	1.57	2.23	3.07	3.92	4.70
Copper	250	2.35	3.75	5.02	5.73	6.48
oxychloride	500	0.77	0.77	1.13	1.55	1.55
	1000	0.6	0.6	0.85	0.95	0.95

Conc= Concentration, cm= centimeter, ppm= Parts per million, hrs= Hours

Table 3. Percent growth inhibition of *Trichoderma* sp. by different chemical fungicides at various concentrations under in vitro condition

Treatments	**Conc (ppm)**	**Growth Inhibition (%)**				
		24 hrs	**36 hrs**	**48 hrs**	**60 hrs**	**72 hrs**
Control		0.00	0.00	0.00	0.00	0.00
Mancozeb	250	32.22^{ef}	27.40^{h}	19.07^{e}	8.89^{i}	0.39^{h}
	500	37.75^{e}	33.08^{fg}	23.20^{e}	13.94^{h}	0.59^{h}
	1000	44.28^{d}	38.13^{de}	28.93^{d}	21.21^{f}	5.88^{g}
Carbendazim	250	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	500	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	1000	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
Hexaconazole	250	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	500	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	1000	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
Carbendazim	250	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
+ Mancozeb	500	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
	1000	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}
Metalaxyl +	250	34.24^{ef}	33.71^{ef}	19.99^{e}	17.37^{g}	5.88^{g}
Mancozeb	500	50.80^{c}	41.29^{d}	31.68^{d}	29.70^{e}	18.24^{f}
	1000	52.81^{c}	57.70^{c}	57.82^{c}	52.53^{d}	44.71^{d}
Copper	250	29.22^{f}	28.98^{gh}	30.99^{d}	30.51^{e}	23.73^{e}
oxychloride	500	76.91^{b}	85.48^{b}	84.41^{b}	81.21^{c}	81.76^{c}
	1000	81.93^{b}	88.64^{b}	88.31^{b}	88.48^{b}	88.82^{b}
Grand Mean		74.45	74.13	71.36	69.10	65
CV (%)		3.74	2.69	2.78	2.09	1.53
LSD (p≤0.01)		6.19	4.42	4.40	3.21	2.21

Conc = Concentration, CV= Coefficient of variation, hrs= hours, LSD= Least significant difference, Same letter in superscripts denote significantly indifferent value (p<0.01)

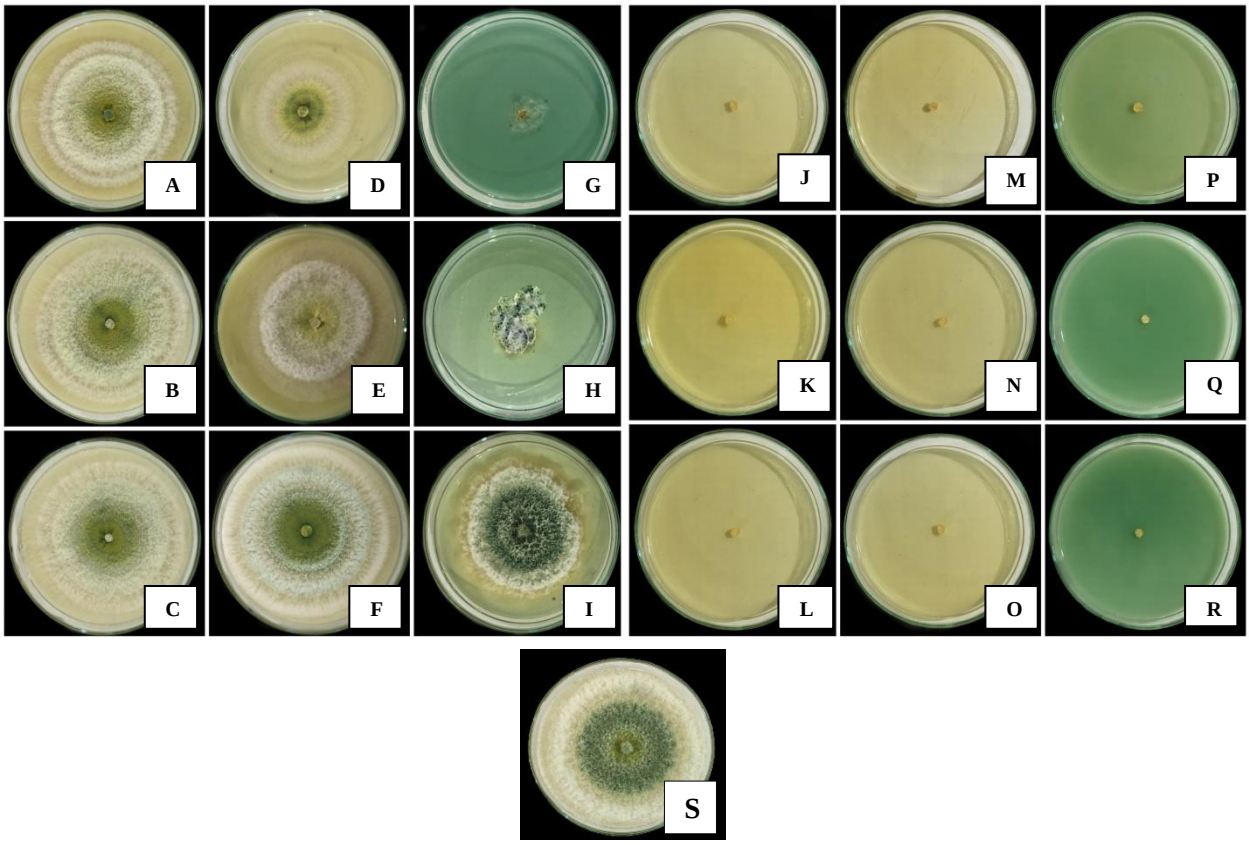

Figure 1. Mycelial growth diameter of Trichoderma sp after 72 hrs of inoculation in media amended with different chemical fungicides at various concentrations – (A) Mancozeb 1000 ppm, (B) Mancozeb 500 ppm, (C) Mancozeb 250 ppm, (D) Metalaxyl+Mancozeb 1000 ppm, (E) Metalaxyl+Mancozeb 500 ppm, (F) Metalaxyl+Mancozeb 250 ppm, (G) Copper oxychloride 1000 ppm, (H) Copper oxychloride 500 ppm, (I) Copper oxychloride 250 ppm, (J) Carbendazim 1000 ppm, (K) Carbendazim 500 ppm, (L) Carbendazim 250 ppm (M) Hexaconazole, 1000 ppm, (N) Hexaconazole 500 ppm, (O) Hexaconazole 250 ppm, (P) Carbendazim+Mancozeb 1000 ppm, (Q) Carbendazim+Mancozeb 500 ppm, (R) Carbendazim+Mancozeb 250 ppm, (S) Control.

For fungicides, Metalaxyl+Mancozeb and Mancozeb, there was a significant increase (<0.01) in GIP as the concentration increased, and with time, there was a gradual decrease in GIP making them more compatible with time, but for Copper oxychloride, it showed variable GIP with time. However, for fungicides Carbendazim, Carbendazim+Mancozeb, and Hexaconazole neither, the GIP decreased with time nor, there was a significant increase in GIP as concentration increased, but throughout the experiment, they showed 100% GIP. The decrease in GIP of fungicides with time, up to our intelligence, is due to the decreased efficacy of the fungicides and the increased ability of the *Trichoderma* sp to neutralize the lethal substance present in those fungicides. Manadhar et al., (2020) and Poudel et al., (2023) also reported that with decreased concentration of fungicides and increased incubation period, the colony diameter of *Trichoderma* sp increased significantly.

Discussion

Out of six fungicides screened under *in-vitro*, Mancozeb at 250 ppm showed the highest compatibility with *Trichoderma* sp. followed by 250 ppm of Metalaxyl+Mancozeb and Copper oxychloride, so these fungicides at lower recommended doses can be used in combination with *Trichoderma* sp. in IDM, whereas irrespective of time and concentrations, Carbendazim, Hexaconazole, and Carbendazim+Mancozeb were utterly incompatible as they completely inhibited the growth, and hence is not recommended to use in IDM. Similar works carried out by Mishra et al., (2019) also reported that among the fungicides tested, Mancozeb 75% WP was most compatible, and at 200 ppm concentration, the recorded GIP was 53.96 % at 48 hrs, but with time, its GIP decreased and reached to 42.96% at 144 hrs of incubation whereas, for systemic fungicides Hexaconazole 5% SC and Carbendizim 50% WP, there was constant 100% growth inhibition even at 100 ppm concentration, observed at 48, 96, and 144 hrs of incubation. Saravanan et al., (2014) also reported the high incompatibility of Carbendazim with *T. viride* as it completely inhibited the mycelial growth, and to some extent, Mancozeb 75% WP can be compatible with antagonists to use in IDM. Similarly, Madhavi et al., (2011) also recorded high incompatibility of *T. viride* with fungicides like Carbendazim 50% WP, Hexaconazole 5% EC, and Carbendazim12%+Mancozeb64%WP, but for Mancozeb 75% WP, *T. viride* was compatible with GIP of 28.29% at 0.25% concentration recorded after 4 days of incubation. Additionally, they reported that contact fungicide, Copper oxychloride 50% WP is incompatible with GIP of 62.9% at the concentration of 0.2%, but the work conducted by Gaur and Sharma (2010) reported that Copper oxychloride 50% WP showed moderate to good compatibility with *T. viride*. Here, in our experiment also, Copper oxychloride was compatible with *T. viride* at a lower concentration (250 ppm), but at a higher concentration (500 and 1000 ppm), it was incompatible. So, the compatibility all depends on concentration.

Several earlier published reports had also mentioned good growth of *Trichoderma* spp at medium and low concentrations of various fungicides (Manandhar et al., 2020, Shashikumar et al., 2019). Dhanya et al., (2016) reported that *T. viride* was sensitive to Carbendazim 50% WP, Hexaconazole 5% EC with extremely high GIP, and for Copper oxychloride 50% WP, they reported GIP of 50.32% at 2g/l. As in our experiment, Nandini et al., (2018) also obtained compatibility between *T. viride* and Metalaxyl + Mancozeb at lower concentrations i.e. 500 ppm recording 19.75% growth inhibition. The high inhibition of Carbendazim, a benzimidazole compound, is due to its binding with β-tubulin of fungal pathogens and disrupting the microtubule dynamic, which ultimately disturbs cell division and may lead to cell death (Zhou et al., 2016). Systematic demethylation inhibitors, present on Hexaconzaole, is the main cause of high inhibition, as it primarily works on the fungus vegetative stage and hinders mycelial development (Khalfallaha et al., 1998). Saaf, being a combined fungicide is a mixture of Carbendzim (12%), and Mancozeb (63%), which has a collective effect of systemic and contact fungicides causing high mycelium inhibition.

Soil-borne pathogens are among the most destructive pathogens in crop production with significant losses globally and aren't able to be controlled solely by agrochemicals. Soil-borne plant pathogens have developed resistance and can persist in the soil for extended periods even without a living host, plant debris, or organic matter. Hence, it is best to integrate *Trichoderma* spp with chemical fungicides for better disease management and to break the resistance of pathogens. Various *Trichoderma* spp have been used alone or in combination with fungicides to control seed and soil-borne diseases in IDM (Samuels 1996). Moreover, *Trichoderma* spp can survive in an environment with some remnant of fungicides and can be produced and multiplied at the farm level; hence can act as the best alternative

to synthetic fungicides (Hetong et al., 2008). However, for field applications with promising results, various factors like climatic adaptability, isolates selection, shelf-life, amount of viable spores, and their ability to colonize after inoculation play a crucial role, so future research must address these issues.

Conclusion

The present finding reveals that Hexaconazole, Carbendazim, and Carbendazim+Mancozeb are incompatible with *Trichoderma* sp while Mancozeb at all concentrations and Copper oxychloride at 250 ppm and Metalaxyl+Mancozeb at 250 and 500 ppm concentration are compatible. Compatible fungicides can be selected to be used in combination with *Trichoderma* sp for IDM in agriculture. The compatibility of chemicals with *Trichoderma* sp decreases with an increase in concentration, so an appropriate amount of chemicals needs to be used while using *Trichoderma* sp in IDM. Integration of *Trichoderma* sp with chemicals provides better and sustainable disease management and reduces the residual effect of chemicals in the environment in long run. However, the field experiment is required to find out the efficacy of those compatible chemicals found in this study in disease management.

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