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# Antioxidants and Anticoccidial Potential of Aqueous Extract from Various Tree Leaves containing Condensed Tannins

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**ABSTRACT**

The present study was conducted to determine the antioxidant and anticoccidial properties of aqueous extract from condensed tannins (CT) containing tree leaves (*Acacia nilotica, Eugenia jambolana, Ficus religiosa, Leucaenea leucocephala* and *Psidium guajava*). The CT content was estimated by using butanol-Hcl method. The CT extracted from various tree leaves in water as solvents and then lyophilized. The antioxidant potential of aqueous extract from various CT sources was evaluated by using multiple *in-vitro* colorimetric methods which include 1,1-Diphenyl-2-picrylhydrazyl (DPPH), total reducing power and hydrogen peroxide assays. Ascorbic acid was used as standard antioxidant in our study. However, anticoccidial efficacy of aqueous extract at different concentration (CT: 1, 2, 3 and 4 mg/ml) from various CT sources was performed using coccidial oocysts sporulation inhibition assay. The DPPH free radical scavenging activity was significantly higher in *P. guajava* as compared to other sources whereas hydrogen peroxide ($H_2O_2$) and reducing power activities were significantly ($P<0.05$) lower in *L. leucocephala* compared to other CT sources. Sporulation inhibition (%) of *Eimeria spp.* was significantly ($P<0.05$) higher in *E. jambolana* followed by *P. guajava, A. nilotica, L. leucocephla* while least in *F.* religiosa. *E. jambolana* and *P. guajava* showed maximum sporulation inhibition activity @ 4 mg/ml. It was concluded that CT extracts of *A. nilotica, E. jambolana, F. religiosa, L. leucocephala and P. guajava* leaves possess the antioxidant and anti-coccidian property and may be eco-friendly sustainable alternative, natural antioxidant, anti-coccidian agent and/ or natural feed additive for organic meat production.

***Keywords****:* Anti-coccidiaa, Antioxidants, Aqueous extract, Condensed tannins, Tree leaves

Coccidiosis is documented as the major protozoan disease of poultry caused by the *Eimeria* and their control strategies have depended mainly on anticoccidial drugs (Allen and Fetterer 2002). These synthetic drugs have played most important role in the effective control of poultry coccidiosis, but, their extensive and indiscriminate uses have resulted in the emergence of drug resistant in coccidian strains (Abbas *et al.*, 2011) and accumulation of drug residues in poultry products.

Moreover, growing interest in global organic food production in recent years primarily due to adverse impact of intensive poultry farming on environment, poultry health as well as consumers concern for food safety restricts use of anti-coccidial drugs in poultry feeds (Nogueira *et al.*, 2009). Thus, alternative environmental friendly sustainable novel strategies are required, which could reduce the exclusive reliance on anti-coccidials drugs either prophylactic or therapeutic treatment. World Health Organization estimated that around 65 - 80% of the world population relies on plant based preparations for maintaining good health and combating diseases (Gurinder and Daljit, 2009). In such situations, there is a rehabilitated understanding in the use of plant secondary metabolites (PSM) especially condensed tannins (CT) for safe, effective and cheap control of coccidiosis in poultry. Various scientists all over the world are now keenly engaged in research into the use of plants/tree leaves and their extracts containing PSMs to fight and reduce the heavy economic losses in poultry industry caused by coccidian protozoa (Williams, 2006).

The CT from tree leaves and leaf meal mixture is becoming preferable and may offer better control than chemical anthelmintics in gastrointestinal parasites (GIP) infected animals (Pathak *et al.*, 2013a, 2014a, b; Singh *et al.*, 2015), effective against each and every developmental stage of GIPs (Pathak *et al.*, 2013b, c) and synthetic anti-coccidials to treat Coccidia in poultry (Abbas *et al.*, 2010; Awais *et al.*, 2011). To date, many plants and tree leaves have been evaluated for possible anthelmintics, anticoccidial and antioxidant properties and compounds isolated from them have shown great potential in the treatment of various infectious diseases (Jovanovic and Simic, 2000). The CT content in tree leaves not only having antiparasitic properties but also having potent natural antioxidant properties. To antioxidants may also satisfy the growing interests of poultry consumers, on condition that they prove to be both effective and safe. A number of natural compounds such as tannins and flavonoides have been reported in literature for their combined antioxidant and anticoccidial effects. Keeping all these beneficial effects in view, it is proposed to explore the effect of aqueous CT extracts from selected tree leave sources as potent antioxidants and anticoccidial warrant investigation.

## MATERIALS AND METHODS

### Sample collection and preparation

Five selected tree leaves were lopped from Faculty premises, R.S. Pura, Jammu and were transported to laboratory in fresh state. The tree leaves were shed dried and then powdered in laboratory mill grinder. Once the tree leaves were processed, they were screened for their chemical composition, presence of CT, their potential sources and then performed *in-vitro* assays to determine antioxidant and anti-coccidial properties of aqueous extract.

### Sample and statistical analysis

Locally available tree leaves were analyzed for their chemical composition as per standard protocol (AOAC, 1995). The extraction and estimation of CTs were done as per butanol-Hcl method (Makkar, 2000). For extraction and estimation of CT, fat and pigment free samples were required otherwise the presence of pigments and fats may hinder CT extraction. Therefore, the pigments and fats were removed by extracting the dried leaves powder with petroleum ether containing 1% glacial acetic acid. The data obtained were subjected to analysis of variance and treatment means were ranked using Duncan's multiple range tests (Snedecor and Cochran, 1994). All the data described under this experimental study were analyzed using SPSS (SPSS version 10.0 for windows).

### Preparation of extract

The aqueous extract of CT from selected tree leaves was prepared in our laboratory. The aqueous extract of CT from different sources was prepared as per the method described by Paolini *et al*. (2004) with slight modification as per Pathak *et al*. (2013b, c). For preparation of aqueous extract of CT, 150g dried powder of each tree leaves were mixed with 1500 ml of distilled water in the flask and boiled for one hour. Then, they were allowed to cool down to 40$^{o}$C and the suspension was filtered through muslin gauze followed by filter paper. The filtrate was kept in deep freezer for 24 hrs, which was then frozen and lyophilized in the freeze drier (CHRIST®: ALPHA 1-4 LSe freeze drier, Sciquip, UK). The lyophilized extracts were kept in the deep freezer at -20$^{o}$C temperature till further used for *in vitro* biological assays.

### Antioxidant property

Locally available tree leaves viz. *Acacia nilotica*, *Eugenia jambolana*, *Ficus religiosa*, *Leucaenea leucocephala* and *Psidium guajava* were evaluated as natural antioxidants by *in-vitro* colorimetric methods.

### DPPH radical scavenging assay

The free radical scavenging activity of various tree extracts was measured in terms of hydrogen donating or radical scavenging ability of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical as per the method described by Braca *et al*. (2001). In this assay, the radical scavenger present in the tree leaves extract decolorized the purple coloured methanolic DPPH solution to yellow due to the reduction of the stable DPPH radicals to diphenyl-pricrylhydrazine in the presence of hydrogen-donating antioxidant (Shon *et al.*, 2003). Briefly, 0.1 ml of plant extracts at various concentrations (0-200 µg/ml) was added

to 3 ml of a 0.002% methanolic solution of DPPH and incubated for 30 min at room temperature. The absorbance at 517 nm was measured against a blank (methanol). A low absorbance of the reaction mixture indicated a high free radical scavenging activity. The radical scavenging activity was calculated using the following formula:

Percentage of inhibition = [(Abs control – Abs sample) / Abs control] × 100

**Hydrogen peroxide scavenging assay**

Hydrogen peroxide scavenging assay of aqueous extract of various CT sources and the standard were assessed based on their hydrogen peroxide scavenging ability by Gulcin *et al*. (2002) and slightly modified by Javanmardi *et al*. (2003). Dissolve 0.5 ml of the standard and each lyophilized extract in phosphate buffer (ph 7.4) and then add 0.6 ml of 4mM hydrogen peroxide solution in PBS (pH 7.4). Absorbance was measured at 230 nm after 10 min against blank solution containing PBS without hydrogen peroxide. Control prepared by replacing sample with phosphate buffer.

Percentage of inhibition = [(Abs control – Abs sample) / Abs control] × 100

**Total reducing power**

Total reducing power assay, on the other hand, is used to test the reducing capability of the CT extracts of tree leaves to convert the potassium ferricyanide ($Fe^{3+}$) complex to form potassium ferrocyanide ($Fe^{2+}$). The potassium ferrocyanide will then react with ferric chloride to form ferrous complex which can absorb maximally at 700nm. Total reducing capacity of CT extracts was determined as per the method described by Oyaizu (1986). One ml of each extract at different concentrations were added with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of (1%) solution of potassium ferricyanide and than incubated in a water bath at 50°C for 20 min followed by the addition of 2.5 ml of trichloroacetic acid (10%). The mixture was centrifuged at 3000 rpm for 10 min. A 2.5 ml aliquot of the upper layer was combined with 2.5 ml of distilled water and 0.5 ml of a (0.1%) solution of ferric chloride. Ascorbic acid was used as positive control.

**Anti-coccidial property**

Anti-coccidial property of CT from *A. nilotica, E. jambolana, F. religiosa, L. leucocephala* and *P. guajava* aqueous extracts were assessed on coccidial oocysts sporulation inhibition. Lyophilized CT extracts of tree leaves were prepared at different concentrations (@ 1, 2, 3 and 4 mg/ml) and their effects were ascertained on oocysts sporulation inhibition of avian *Eimeria spp*.

**Isolation and counting of coccidian oocysts**

The poultry droppings were collected in plastic bags from nearby poultry farms which were suspected for avian coccidiosis. Collected samples were kept in ice container and brought to the laboratory for examination. *Eimeria* oocysts positive samples were processed for collection and separation of oocysts as per standard procedure.

**Sporulation inhibition assay**

Sporulation inhibition bioassay was performed to evaluate the anti-coccidial property of CT extracts. Briefly, About 500 un-sporulated *Eimeria* oocysts per ml were adjusted in each well from original aqueous suspension of faeces and then they were exposed to four concentrations of each lyophilised extract of each and every CT source @ 0, 1, 2 and 4 mg/ml (w/v). Three replications were made for each concentration of each and every extract and the whole experiment was repeated three times to confirm the results. In this assay, the un-sporulated oocysts were incubated with different CT source extracts for 48-72 hrs at 28–30 °C temperature in a previously adjusted incubator to maintain constant temperature. The oocysts were gently aerated with an air with the help of pipette away from sun light. At the end of the incubation, the incubated suspensions were washed twice in tap water and stored at 4°C until being counted. Numbers of sporulated and un-sporulated oocysts were counted and the percent sporulation was estimated by counting the number of sporulated oocysts in a total of 100 oocysts. In addition, the numbers of sporocysts within each sporulated oocyst of *Eimeria spp.* were counted and the numbers of abnormal sporocysts were counted. Moreover, oocysts with 4 sporocysts were considered sporulated regardless the shape and size of the sporocysts.

## RESULTS AND DISCUSSION

### Chemical composition

The chemical compositions of locally available tanniferous tree leaves are summarized in table 1. Chemical composition and CT content of tanniferous tree leaves showed wide variation. The chemical composition of target tree leaves in the present study was comparable with the values reported by many workers (Dey *et al.*, 2006; Pathak *et al.*, 2015; Singh *et al.*, 2015), except some species/ nutrient specific differences usually observed in such tree leaves. The CT content varied greatly among tree leaves which are in conformity with the values reported by earlier workers (Pathak, 2013; Pathak *et al.*, 2015; Singh *et al.*, 2015).

**Table 1: Chemical composition of locally available tree leaves (g/kg DM)**

| Attributes | Acacia nilotica | Eugenia jambolana | Ficus religiosa | Leucaena leucocephala | Psidium guajava |
|---|---|---|---|---|---|
| Organic matter | 929.60 | 909.40 | 899.60 | 900.40 | 919.60 |
| Crude protein | 87.40 | 91.10 | 100.70 | 186.30 | 90.20 |
| Ether extract | 46.60 | 45.80 | 35.30 | 49.60 | 40.80 |
| Crude fibre | 90.30 | 124.40 | 115.20 | 82.60 | 122.90 |
| Condensed tannins | 21.60 | 80.60 | 20.10 | 44.70 | 84.90 |

### Antioxidants property

#### *DPPH free radical scavenging activity*

The DPPH free radical scavenging activities of aqueous extract from various tree leaves are presented in the table 2. The DPPH assay has been used widely to determine radical scavenging activity of antioxidant substances (Zhang and Lin, 2008; Zhang *et al.*, 2010). The DPPH free radical scavenging activity was significantly ($P<0.05$) higher in *P. guajava* followed by *E. jambolana*, *A. nilotica* and *F. religiosa*, while *L. leucocephala* was having least DPPH free radical scavenging activity. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H (Dinis *et al.*, 1994; Singleton *et al.*, 1999). The CT significantly inhibited the activity of DPPH radicals in a dose-dependent manner and the maximum scavenging activities were observed at the concentration of 200 mg per ml.

**Table 2: DPPH free radical scavenging activity of aqueous extracts from various CT sources in comparison to ascorbic acid**

| CT Sources | DPPH % Dilution (µg/ml) | | | | |
|---|---|---|---|---|---|
| | 25 | 50 | 100 | 150 | 200 |
| *A. nilotica* | $10.50^{Ab}$ | $17.00^{Bb}$ | $30.23^{Cb}$ | $51.40^{Db}$ | $58.17^{Eb}$ |
| *E. jambolana* | $11.40^{Abc}$ | $19.93^{Bbc}$ | $31.80^{Cbc}$ | $52.50^{Dbc}$ | $59.50^{Ebc}$ |
| *F. religiosa* | $13.27^{Ab}$ | $21.97^{Bb}$ | $29.77^{Cb}$ | $42.37^{Db}$ | $50.93^{Eb}$ |
| *L. leucocephala* | $6.60^{Aa}$ | $7.50^{Ba}$ | $8.60^{Ca}$ | $9.24^{Da}$ | $11.63^{Ea}$ |
| *P. guajava* | $16.05^{Ac}$ | $22.73^{Bc}$ | $37.30^{Cc}$ | $53.07^{Dc}$ | $61.73^{Ec}$ |
| Ascorbic acid | 22.98 | 53.28 | 94.13 | 97.08 | 97.96 |

$^{ABCDE}$ & $^{abc}$ Means with different superscript with in a row and column differ significantly ($P<0.05$)

The higher radical scavenging activity observed in *P. guajava* leaves is perhaps attributed to the higher CT content in these leaves. In the present study, the CT content and the radical scavenging activity of *P. guajava* leaves are likely to showed good relationship. Previous studies had also reported the relationship between the high level of polyphenolic compounds and radical scavenging activity (Bertoncelj *et al.*, 2007; Park *et al.*, 2008). On the other hand, the higher DPPH free radical scavenging activity of *P. guajava* extracts may be due to the potential and effective CT source, because of reaction between CT molecules and radicals, resulted in the scavenging of radicals by hydrogen donation (Ogawa *et al.*, 2008).

#### *Hydrogen peroxide scavenging activity*

Scavenging of $H_2O_2$ of CT extracts from selected tree leaves are presented in table 3. In the present study, aqueous extract of *L. leucocephla* showed the least $H_2O_2$ scavenging activity compared to other CT sources while *E. jambolana* was having highest $H_2O_2$ scavenging activity. Even though, *P. guajava* had an intermediate value between *F. religiosa*, *A. nilotica* and *E. jambolana*. The $H_2O_2$ scavenging activity was significantly higher at 50 µg/ml concentration as compared to other concentrations. Although, $H_2O_2$ not a radical species which play a role to contribute oxidative stress. The generation of even low

levels of $H_2O_2$ in biological systems may be important. $H_2O_2$ itself is not very reactive, but can sometimes be toxic to cell (Miller *et al.,* 2000) because naturally occurring iron complexes inside the cell believed to react with $H_2O_2$ and it may give rise to hydroxyl radical in the cells (Halliwell *et al.,* 1987). Thus, removal of $H_2O_2$ is very important for protection of food systems. Scavenging of $H_2O_2$ by extracts may be attributed to their CT content, which can donate electrons to $H_2O_2$, thus neutralizing it to water. The CT extracts were capable of scavenging $H_2O_2$ in a concentration-dependent manner.

**Table 3: Hydrogen peroxide scavenging activity of aqueous extracts from various CT sources in comparison to ascorbic acid**

| CT Source | Hydrogen peroxide Dilution (µg/ml) | | | | |
|---|---|---|---|---|---|
| | 10 | 20 | 30 | 40 | 50 |
| *A. nilotica* | $9.40^{Ab}$ | $16.20^{Bb}$ | $26.20^{Cb}$ | $37.49^{Db}$ | 44.83Eb |
| *E. jambolana* | $12.80^{Ac}$ | $20.10^{Bc}$ | $31.70^{Cc}$ | $40.00^{Dc}$ | $48.00^{Ec}$ |
| *F. religiosa* | $9.90^{Ab}$ | $17.30^{Bb}$ | $26.30^{Cb}$ | $37.70^{Db}$ | $45.80^{Eb}$ |
| *L. leucocephala* | $5.57^{Aa}$ | $7.49^{Ba}$ | $9.90^{Ca}$ | $18.40^{Da}$ | $25.80^{Ea}$ |
| *P. guajava* | $11.10^{Abc}$ | $17.80^{Bbc}$ | $28.00^{Cbc}$ | $38.70^{Dbc}$ | $46.80^{Ebc}$ |
| Ascorbic acid | 44.83 | 55.33 | 62.30 | 71.58 | 80.83 |

$^{ABCDE\&abc}$ Means with different superscript with in a row and column differ significantly (P<0.05)

## Reducing power assay

The reducing capacity of CT extracts from various tree leaves are presented in the table 4. In reducing power assay, a higher absorbance indicates a stronger reducing power. The *A. nilotica* showed significantly (P<0.05) higher reducing ability compared to other CT sources. The reducing capacity of tree leaves extracts is much related to the presence of biologically active compounds (CT) with potent donating abilities may therefore, serve as an indicator of its potential antioxidant activity (Elzaawely *et al.,* 2005). In the present study we observed a concentration-dependent decrease in the absorbance of reaction mixture for all CT sources and ascorbic acid. The observed reducing ability of *A. nilotica*, *E. jambolana* and *P. guajava* extracts in the present study could be attributed to the presence of CT as it also reported by Omoruyi *et al*. (2012). Previous studies of Omoruyi *et al*. (2012) and Park and Jhon (2010) correlated the reducing power ability of plant extracts with the presence of phenolic content. The antioxidant potential and effectiveness of CT is generally proportional to the number of hydroxyl (–OH) groups present on the aromatic ring (s) as well as arrangement of the hydroxyl groups and extraction processes.

**Table 4: Reducing power activity of aqueous extracts from various CT sources in comparison to ascorbic acid**

| CT Source | Dilution Rate (µg\ml) | | | | |
|---|---|---|---|---|---|
| | 20 | 40 | 60 | 80 | 100 |
| *A. nilotica* | $0.14^{Ae}$ | $0.20^{Be}$ | $0.26^{Ce}$ | $0.32^{De}$ | $0.39^{Ee}$ |
| *E. jambolana* | $0.10^{Ac}$ | $0.19^{Bc}$ | $0.21^{Cc}$ | $0.27^{Dc}$ | $0.31^{Ec}$ |
| *F. religiosa* | $0.07^{Ab}$ | $0.10^{Bb}$ | $0.11^{Cb}$ | $0.14^{Db}$ | $0.20^{Eb}$ |
| *L. leucocephala* | $0.02^{Aa}$ | $0.04^{Ba}$ | $0.05^{Ca}$ | $0.07^{Da}$ | $0.09^{Ea}$ |
| *P. guajava* | $0.15^{Ad}$ | $0.19^{Bd}$ | $0.24^{Cd}$ | $0.29^{Dd}$ | $0.34^{Ed}$ |
| Ascorbic acid | 0.32 | 0.47 | 0.54 | 0.66 | 0.77 |

$^{ABCDE\&abcde}$ Means with different superscript with in a row and column differ significantly (P<0.05)

## Anti-coccidial property

The effect of CT extracts from various sources on sporulation and sporulation inhibition of *Eimeria* oocysts are presented in the table 5 and 6. The percentage sporulation inhibition of *Eimeria spp.* was significantly (P<0.05) higher in *E. jambolana* and *P. guajava* followed by *A. nilotica* and least in *L. leucocephla* and *F. religiosa*. As the CT concentration (mg/ml) increased the sporulation inhibition drastically reduced and the maximum sporulation inhibition was recorded at 4 mg/ml.

**Table 5: Effect of aqueous extracts from various CT sources on % oocysts soprulation of avian *Eimeria spp*.**

| Leaf source | Control | CT concentrations (mg/ml) | | | | Mean ± SE |
|---|---|---|---|---|---|---|
| | | 1.00 | 2.00 | 3.00 | 4.00 | |
| *A. nilotica* | 88.00 | 73.33 | 58.67 | 53.33 | 47.00 | $58.08^{b}$ ±3.03 |
| *E. jambolana* | „ | 76.67 | 60.33 | 46.33 | 35.67 | $54.75^{a}$ ±4.75 |
| *F. religiosa* | „ | 80.33 | 77.33 | 72.33 | 68.33 | $74.58^{c}$ ±1.46 |
| *L. leucocephala* | „ | 78.33 | 76.00 | 71.33 | 66.67 | $73.08^{c}$ ±1.53 |
| *P. guajava* | „ | 72.33 | 58.33 | 51.00 | 38.67 | $55.08^{a}$ ±3.76 |
| Mean ± SE | | $76.20^{d}$ ±0.93 | $66.13^{c}$ ±2.44 | $58.87^{b}$ ±2.97 | $51.27^{a}$ ±3.77 | |

$^{abcd}$ Means with different superscript with in a row and column differ significantly (P<0.05)

**Table 6: Effect of aqueous extract from various CT sources on % sporulation inhibition of avian *Eimeria spp.***

| Leaf source | CT concentration (mg/ml) | | | | Mean ± SE |
|---|---|---|---|---|---|
| | 1.00 | 2.00 | 3.00 | 4.00 | |
| *A. nilotica* | 16.67 | 33.31 | 39.38 | 46.60 | $33.99^{b}$ ±3.45 |
| *E. jambolana* | 12.86 | 31.46 | 47.34 | 59.48 | $37.78^{c}$ ±5.40 |
| *F. religiosa* | 8.69 | 12.10 | 17.80 | 22.35 | $15.24^{a}$ ±1.70 |
| *L. leucocephala* | 10.99 | 13.62 | 18.94 | 24.25 | $16.95^{a}$ ±1.72 |
| *P. guajava* | 17.79 | 33.70 | 42.05 | 56.04 | $37.39^{c}$ ±4.28 |
| Mean ± SE | $13.40^{a}$ ± 1.08 | $24.84^{b}$ ± 2.79 | $33.10^{c}$ ± 3.38 | $41.74^{d}$ ± 4.27 | |

$^{abcd}$ Means with different superscript with in a row and column differ significantly (P<0.05)

The *E. jambolana* and *P. guajava* CT extract showed maximum sporulation inhibition activity and effective against *Eimeria* oocysts. Similar to present findings, Molan *et al*. (2009a) also observed *in-vitro* sporulation inhibition against aqueous extracts of pine bark (*Pinus radiata*) in three species of avian coccidia. The mechanism by which CT extracts inhibited the sporulation process is not clear but CT can inhibit the activities of various endogenous enzymes (Horigome *et al.*, 1988). This encouraged us to speculate that the CT extracts of various tree leaves may inhibit the enzymes responsible for the sporulation process of the coccidian oocysts (Molan *et al.*, 2009b). Jones *et al*. (1994) suggested that CT may penetrate the cell wall of bacteria and cause a loss of intracellular components. In the present study, the CT extracts may penetrate the wall of the oocyst and damage the cytoplasm (sporont) as evidenced by the appearance of abnormal sporocysts in oocyst exposed to CT extracts.

## CONCLUSION

It may be concluded that CT containing tree leaves act as eco-friendly sustainable natural antioxidants and anti-coccidian agent to control avian coccidiosis. The CT extracts of *A. nilotica*, *E. jambolana*, *F. religiosa*, *L. leucocephala and P. guajava* leaves possess antioxidants and anticoccidial properties, however, *E. jambolana* and *P. guajava* were found to be most potent CT sources as well as natural antioxidant and anticoccidial agent.

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