Pharmacological and Toxicological Study of *Maytenus ilicifolia* Leaf Extract Part II—Clinical Study (Phase I)

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Maytenus ilicifolia is a plant widely used in South American folk medicine as an effective anti-dyspeptic agent, and the aim of this study was to evaluate their clinical and toxicological effects in healthy volunteers in order to establish its maximum safe dose. We selected 24 volunteers (12 women and 12 men) between 20 and 40 years of age and put them through clinical/laboratory screening and testing to ascertain their psychomotor functions (simple visual reaction, speed and accuracy, finger tapping tests). *M. ilicifolia* tablets were administered in increasing weekly dosages, from an initial dose of 100 mg to a final dose of 2000 mg. The volunteers’ clinical and biochemical profiles and psychomotor functions were evaluated weekly, and they also completed a questionnaire about any adverse reactions. All subjects completed the study without significant changes in the evaluated parameters. The most cited adverse reactions were xerostomia (dry mouth syndrome) (16.7%), and polyuria (20.8%), with reversal of these symptoms without any intervention during the study. The clinical Phase I study showed that the administration of up to 2000 mg of the extract was well tolerated, with few changes in biochemical, hematological or psychomotor function parameters, and no significant adverse reactions. Copyright © 2017 John Wiley & Sons, Ltd.

Keywords: clinical research; anti-ulcer effect; safety; Maytenus; serological and biochemical data.

INTRODUCTION

*M. ilicifolia* Mart ex Reis (popularly known as espinheira santa) is a plant widely used in folk medicine as an effective anti-dyspeptic agent (Niero et al., 2011). The leaves of this species have been shown to have a beneficial action on gastric ulcers in animals, with freeze dried extract having been shown to have a proven protective effect against the development of ulcers in laboratory rats, both orally and intraperitoneally, comparable in effect to cimetidine (Carlini, 1988; Souza-Formigoni et al., 1991; Niero et al., 2011).

We also evaluated any possible toxic effects, with no changes being observed in weight, behavior, temperature or in serum and hematological biochemical parameters in laboratory animals, no changes were observed in either the estrous cycle of rats or the reproductive capacity of males and females, and pups born to mothers who received the extract developed normally (Oliveira et al., 1991; Tabach et al., 2017—PTR-16-1269).

In the previous Phase I clinical pilot study (Carlini and Frochtengarten, 1988), seven healthy volunteers took *M. ilicifolia* for 14 days at a dosage corresponding to twice the dose commonly used in folk medicine. Both at the 7th and the 14th day, there were no abnormal results or adverse effects observable by ECG, physical examination, hematological and serum biochemical analyses and urine tests.

This study was followed by a Phase II pilot study of patients with non-ulcer dyspepsia, who received 400 mg of freeze-dried plant for 28 days in a double-blind trial. Treated patients showed statistically significant improvements in overall dyspeptic symptoms and in the symptoms of heartburn, pain and nausea (Geocze et al., 1988).

These promising preliminary results led to the continuation of studies with the preparation of a new extract of *M. ilicifolia*, stable and with adequate quality control, through a partnership between Cebrid/Unifesp and Aché Laboratórios farmacêuticos S.A. This preparation led to a patent (PI 9904502-8).

Preclinical studies in rats performed in our laboratories with this extract show that administration of up to 280 mg/kg of this preparation protected the gastric mucosa against the development of ulcers caused by stress induced by immobilization at low temperature. In addition, there was an increase in the volume and pH of gastric juice, and prolonged administration for three months in the laboratory animals did not cause significant changes relating to toxicity, mutagenicity or teratogenicity (Tabach et al., 2017—PTR-16-1269).
These positive results were the basis for this clinical study with the objective of establishing a maximum safety range in the use of tablets obtained from this new pharmaceutical preparation from *M. ilicifolia*.

**MATERIALS AND METHODS**

**Study design.** The study protocol was designed with increasing doses, spaced a week apart, using tablets prepared from the standardized extract of *M. ilicifolia* (MIE). The study was conducted according to the standards of the Declaration of Helsinki (1996) and was reviewed and approved by the Ethics Committee of the Federal University of São Paulo (process 563/99).

**Preparation and evaluation of the extract.** Leaves of *M. ilicifolia* grown in Centro Pluridisciplinar de Pesquisas Químicas, Biológicas e Agronômicas of the Universidade de Campinas (Unicamp) were dried and powdered. A voucher specimen was deposited in Unicamp Herbarium (UEC 112745). This material (3 g) was subjected to extraction with 150 mL of water heated between 85 and 100°C for 15 min by infusion. The determinations of total phenolic and tannin in the extract were performed by Folin–Dennis method as previously described by AOAC (1995). The dry extract had a yield of 17.5%, residual moisture of 3.96%, total polyphenol content of 41.57% and tannin fraction of 17.96%. The extract was filtered and spray-dried according to the method described by Souza-Fornigoni et al. (1991). The extraction solution was separated from the plant by pressing material. The dry extract of *M. ilicifolia* was obtained by spray drying tower from the aqueous extraction solution containing 20% of highly dispersed silicon dioxide (Aerosil 200®/Degussa) under the following conditions: inlet temperature—146°C; outlet temperature—97°C; feed stream—3 mL/min and spray pressure—2 bar.

**Inclusion and exclusion criteria.** Twenty-four volunteers were selected and remained throughout the experimental protocol, completing the study, as detailed in Fig. 1. **Inclusion:** Healthy volunteers aged between 20 and 40 years were selected (Table 1)—their health checked through clinical and psychiatric evaluation, ECG and urine analysis, and by checking their biochemical and hematological parameters. All volunteers agreed to follow the experimental protocol and signed the Terms of Informed and Free Consent (TECLE), which explained the aims and methods of the research and ensured the right to leave the study at any time, if they so wished.

**Exclusion:** chronic use of medication (with the exception of contraceptives); history of intolerance to plant products; pathological history—particularly from cardiovascular, hepatic, renal, pulmonary, endocrinal or allergic causes; smoking (more than five cigarettes a day); alcoholism; use of illicit drugs.

**Table 1.** Baseline characteristics of the individuals included in the study

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>26.3 ± 4.3</td>
<td>27.6 ± 4.6</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.8 ± 9.5</td>
<td>58.3 ± 9.5</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.74 ± 0.06</td>
<td>1.65 ± 0.06</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.6 ± 2.5</td>
<td>21.4 ± 2.9</td>
</tr>
<tr>
<td>Systolic pressure (mmHg)</td>
<td>124.2 ± 11.6</td>
<td>118.8 ± 3.1</td>
</tr>
<tr>
<td>Diastolic pressure (mmHg)</td>
<td>81.7 ± 5.8</td>
<td>78.8 ± 3.1</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>78.5 ± 4.1</td>
<td>73.2 ± 3.7</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation.

![Figure 1](https://example.com/f1.png)

**Figure 1.** Flow chart of the volunteers who participated in the clinical trial (Obs.: MIE: *Maytenus ilicifolia* extract; W: week).
Procedure and choice of doses. Once selected, the volunteers were subjected to a protocol (Fig. 1) which is repeated over 6 weeks of treatment. It involved increasing the dose at weekly intervals. The experiment was conducted without a control group, with the results of the initial tests performed during the selection process being considered as a baseline for comparison. The different doses, in increasing order, were administered once only, with the initial dose set at 100 mg per adult with subsequent doses of 200, 500, 1000, 1500 and 2000 mg, so at a much lower dose than the minimum which has been found to be toxic to animals (Tabach et al., 2017—PTR-16-1206). This study was based on an analysis of how this plant is popularly used, which showed a dose equivalent to 7 mg/kg in humans. Therefore, the specific therapeutic dose would be 490 mg for a 70-kg person. Throughout the study, and before taking the different doses of MIE, weekly blood and urine samples were obtained for biochemical/hematological analysis. In addition to these laboratory tests, the volunteers were subjected to weekly clinical and psychiatric evaluations, physical exams and psychomotor assessment tests.

Blood collection for hematological and biochemical tests. Samples of blood and urine were obtained from the volunteers at the start of the screening process (time 0) and weekly throughout the study (W1–W6) for assessment of vital functions (liver, kidney, lipid profile, glucose), and a white and red blood cell count. After completion of the study, two more collections were carried out, one in the first week and the other in the fourth week, and on these two occasions the volunteers also underwent a detailed clinical evaluation.

Neuropsychomotor evaluation

Simple visual reaction test (alert test). This test is performed by means of a program that produces a visual stimulus on a computer screen, which the volunteer responds to by pressing the mouse (Bracy, 1995). Reaction time indicates the volunteer’s alertness, which is ‘the time interval between a given stimulus and the response of the volunteer.’ We evaluated the average length of the reactions (average of 15 repetitions).

Speed and accuracy test. The visual-motor test proposed by Sack and Rice (1974), with minor modifications, was performed using a sheet of 400 numbers (0 to 9) arranged in lines. The volunteers were instructed to mark one of the numbers (N° 4, for example) as quickly as possible. Speed and accuracy were evaluated using the total time taken to complete the test and the number of correct and incorrect answers.

Finger tapping test (FTT). This test indirectly assesses the motor speed of the upper limbs and was adapted from Strauss et al. (2006). It consisted of a bar connected to a digital counter. The volunteers were instructed to hit the bar with the index finger of their dominant hand as rapidly as possible for 1 min, and the total number of hits was recorded for evaluation of performance.

Adverse reactions. After the psychomotor evaluation, the volunteers completed a questionnaire about any adverse drug reactions experienced during the procedure.

Statistical analysis. The evaluation of the biochemical and hematological parameters of the volunteers’ blood, and the results of the psychomotor evaluations obtained during the study were compared with those obtained from the volunteers at the start (time zero) and were within the normal range for each individual to be considered healthy. A two-way analysis of variance (ANOVA) was used, followed by Duncan test a posteriori when necessary.

RESULTS

Neuropsychological assessment

The results revealed that the administration of increasing doses of MIE did not cause significant changes over time in all parameters evaluated by the different tests.

Simple visual reaction test. An improvement in performance was observed in the alert test compared with time zero throughout the administration of the MIE. However, this effect remained in the post study evaluation, one week after the end of treatment, indicating that it was more a phenomenon resulting from learning the task.

The speed and accuracy test. There was a statistically significant reduction (p < 0.01) in the time required to find the target numbers from the third evaluation session (500 mg), compared with baseline. However, this reduction also remained in the post study evaluation. Thus, we conclude that this was probably the result of the learning process. In respect of the numbers not found, there was no statistically significant difference throughout the study (data not shown).

The finger tapping test. No significant change in this parameter over the entire treatment was observed, indicating that the MIE did not change the volunteers’ alertness (data not shown).

Adverse reactions. There were no significant changes in the evaluated parameters in both male and female volunteers throughout the study. There were some sporadic adverse reactions, but they did not entail discontinuation of treatment. The most common are described in Table 2.

The most common adverse reactions were polyuria, which was observed in five patients (20.85%), most often in the fourth and fifth weeks of treatment. All patients had spontaneous remission of this condition over time. Xerostomia (having a dry mouth) was the
second most frequent adverse reaction (16.7%) that, as with polyuria, had spontaneous remission.

Hematological and biochemical tests. The results revealed that administration of up to 2000 mg of MIE did not cause significant changes in the normal values of the main hematological and biochemical parameters (Table 3).

As can be seen, all parameters remained within normal limits, both in the male and in the female volunteers. The values remained within normal parameters in post-treatment tests performed four weeks after the end of the administration of MIE.

Table 4 shows that creatinine serum levels, uric and urea did not change during the treatment and, therefore, had no influence on renal function. Similarly, liver function did not change significantly, because the liver enzymes (AST, ALT) remained within normal values.

Finally, all the remaining parameters (glucose, potassium, albumin, HDL, VLDL and LDL, cholesterol and triglyceride) showed no significant changes during the treatment, as can be seen in Table 4. In the post-treatment phase, all results were also within normal values.

DISCUSSION

The MIE extract showed to be safe until a dose of 2000 mg/day in healthy volunteers as well as in preclinical studies performed in our labs (Tabach et al., 2017—PTR-16-1269). Considering different experimental models using mice, rats and dogs, the MIE did not cause deaths or any significant change in the behavioral, anatomical or pathological parameters of animals. Therefore, the extract has a promising potential as a relatively safe and non-toxic drug with demonstrated anti-dyspeptic activity, reducing acidity, heartburn and nausea in humans.

Although *M. ilicifolia* is a well-known plant in South America and its use widespread in the population, the analysis of the literature revealed a large number of pre-clinical studies (Carlini, 1988; Cipriani et al., 2009; Leite et al., 2010; Oliveira et al., 1991; Souza-Formigoni et al., 1991; Tabach and Oliveira, 2003; among others), but relatively few clinical trials (Carlini and Frochtengarten, 1988; Goecke et al., 1991).

The results from this clinical study show its advantages compared with the synthetic proton pump inhibitors used in the treatment of gastritis and ulcers such as omeprazole, which has several side effects, including decreased absorption of vitamin B12, and an increase in bone fractures, among others (Dobrowski et al., 2013; Lam et al., 2013).

The potential benefits of a plant-based preparation should be assessed alongside standard products, which must be tested both in preclinical and clinical studies while taking into consideration its traditional or popular use. The initial dose in this study was 100 mg of MIE a day and was successively raised to 2000 mg/day.

The analysis of liver, kidney and pancreatic functions, as well as cholesterol levels, glucose, hormones and the results of type I urine examination showed that these parameters remained within normal values throughout the treatment, including at the highest dose (2000 mg) used in this study.

The adverse events reported by the volunteers were few and not of a serious nature and did not, therefore, stop treatment. The most common were related to the renal system (polyuria 5/24) and the digestive system (xerostomia 4/24). However, clinical and laboratory tests (blood and urine) performed not only in this intervention. The other most common adverse reaction reported by volunteers was xerostomia (dry mouth) which is relatively common and usually due to the nervousness, stress or sickness. This reaction may be related also to the presence of tannin, because these

### Table 3. Hematological parameters of the 24 volunteers treated with *Maytenus ilicifolia* extract for 6 weeks

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Pre-treatment</th>
<th>Week 1</th>
<th>Week 3</th>
<th>Week 6</th>
<th>Post-treatment</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes (10^6 L⁻¹)</td>
<td>4.77 ± 0.41</td>
<td>4.75 ± 0.44</td>
<td>4.84 ± 0.36</td>
<td>4.65 ± 0.54</td>
<td>4.84 ± 0.43</td>
<td>4.50–6.00</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>14.03 ± 1.48</td>
<td>13.64 ± 1.27</td>
<td>13.77 ± 1.16</td>
<td>13.48 ± 1.70</td>
<td>13.78 ± 1.65</td>
<td>12.00–18.00</td>
</tr>
<tr>
<td>Platelets (10^9 L⁻¹)</td>
<td>240.04 ± 36.82</td>
<td>245.88 ± 51.19</td>
<td>241.46 ± 43.38</td>
<td>256.88 ± 51.46</td>
<td>269.57 ± 52.51</td>
<td>140.00–400.00</td>
</tr>
<tr>
<td>Leukocytes (10^9 L⁻¹)</td>
<td>6.10 ± 1.21</td>
<td>6.00 ± 1.10</td>
<td>6.56 ± 1.37</td>
<td>6.72 ± 1.25</td>
<td>6.39 ± 1.31</td>
<td>3.60–10.70</td>
</tr>
<tr>
<td>Neutrophils (10^9 L⁻¹)</td>
<td>3.54 ± 1.12</td>
<td>3.32 ± 0.98</td>
<td>3.69 ± 0.93</td>
<td>3.78 ± 1.16</td>
<td>3.70 ± 1.22</td>
<td>1.80–7.00</td>
</tr>
<tr>
<td>Eosinophils (10^9 L⁻¹)</td>
<td>0.22 ± 0.20</td>
<td>0.24 ± 0.20</td>
<td>0.25 ± 0.21</td>
<td>0.31 ± 0.37</td>
<td>0.25 ± 0.24</td>
<td>0.00–0.50</td>
</tr>
<tr>
<td>Basophils (10^9 L⁻¹)</td>
<td>0.05 ± 0.02</td>
<td>0.05 ± 0.02</td>
<td>0.06 ± 0.02</td>
<td>0.06 ± 0.03</td>
<td>0.08 ± 0.03</td>
<td>0.00–0.20</td>
</tr>
<tr>
<td>Lymphocytes (10^9 L⁻¹)</td>
<td>1.79 ± 0.60</td>
<td>1.87 ± 0.57</td>
<td>1.98 ± 0.65</td>
<td>2.11 ± 0.75</td>
<td>1.90 ± 0.68</td>
<td>1.00–4.30</td>
</tr>
<tr>
<td>Monocytes (10^9 L⁻¹)</td>
<td>0.37 ± 0.10</td>
<td>0.38 ± 0.12</td>
<td>0.42 ± 0.09</td>
<td>0.42 ± 0.15</td>
<td>0.34 ± 0.09</td>
<td>0.00–0.90</td>
</tr>
</tbody>
</table>

Note: The treatment was performed with increasing weekly doses; 100 mg (Week 1), 200 mg, 500 mg, 1000 mg, 1500 mg and 2000 mg (Week 6). Values are means ± standard deviation.

Table 4. Biochemical parameters of 24 volunteers during 6-week treatment with Maytenus ilicifolia extract

<table>
<thead>
<tr>
<th></th>
<th>Pre-treatment</th>
<th>Week 1</th>
<th>Week 3</th>
<th>Week 6</th>
<th>Post-treatment</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>84.63 ± 7.31</td>
<td>82.71 ± 8.82</td>
<td>87.13 ± 8.61</td>
<td>91.38 ± 9.24</td>
<td>84.65 ± 7.50</td>
<td>60.00–110.00</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.93 ± 0.22</td>
<td>0.89 ± 0.18</td>
<td>0.85 ± 0.13</td>
<td>0.82 ± 0.13</td>
<td>0.87 ± 0.14</td>
<td>0.60–1.20</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>4.29 ± 1.10</td>
<td>4.65 ± 1.39</td>
<td>4.43 ± 1.21</td>
<td>4.43 ± 1.48</td>
<td>4.51 ± 1.68</td>
<td>3.50–7.40</td>
</tr>
<tr>
<td>Potassium (mg/dL)</td>
<td>4.13 ± 0.25</td>
<td>4.07 ± 0.24</td>
<td>4.10 ± 0.28</td>
<td>4.06 ± 0.27</td>
<td>4.10 ± 0.27</td>
<td>2.50–7.55</td>
</tr>
<tr>
<td>Urea (mg/dL)</td>
<td>27.13 ± 7.36</td>
<td>29.29 ± 6.96</td>
<td>25.96 ± 6.10</td>
<td>26.92 ± 6.14</td>
<td>28.04 ± 7.02</td>
<td>16.00–40.00</td>
</tr>
<tr>
<td>Albumin (mg/dL)</td>
<td>3.88 ± 0.53</td>
<td>4.45 ± 0.49</td>
<td>4.51 ± 0.43</td>
<td>4.51 ± 0.30</td>
<td>4.44 ± 0.42</td>
<td>3.50–5.50</td>
</tr>
<tr>
<td>GOT (mg/dL)</td>
<td>21.00 ± 6.16</td>
<td>24.58 ± 12.83</td>
<td>23.54 ± 7.49</td>
<td>23.92 ± 8.89</td>
<td>26.91 ± 14.81</td>
<td>0.00–30.00</td>
</tr>
<tr>
<td>GPT (mg/dL)</td>
<td>16.75 ± 7.02</td>
<td>23.83 ± 16.10</td>
<td>21.25 ± 19.77</td>
<td>17.13 ± 8.65</td>
<td>19.30 ± 12.85</td>
<td>0.00–40.00</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>55.21 ± 11.91</td>
<td>53.92 ± 13.97</td>
<td>49.17 ± 6.22</td>
<td>± 12.56</td>
<td>56.83 ± 12.15</td>
<td>&gt;40.00</td>
</tr>
<tr>
<td>VLDL (mg/dL)</td>
<td>14.78 ± 4.71</td>
<td>15.03 ± 6.64</td>
<td>16.64 ± 7.90</td>
<td>20.08 ± 21.15</td>
<td>14.55 ± 6.51</td>
<td>&lt;30.00</td>
</tr>
<tr>
<td>LDL (mg/dL)</td>
<td>93.98 ± 32.30</td>
<td>100.33 ± 33.28</td>
<td>102.53 ± 33.87</td>
<td>101.23 ± 30.83</td>
<td>103.28 ± 36.15</td>
<td>&lt;130.00</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>163.96 ± 33.40</td>
<td>169.29 ± 37.76</td>
<td>173.96 ± 37.47</td>
<td>171.79 ± 34.66</td>
<td>166.91 ± 52.76</td>
<td>&lt;200.00</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>73.88 ± 23.54</td>
<td>75.13 ± 33.22</td>
<td>85.21 ± 39.49</td>
<td>79.54 ± 35.58</td>
<td>72.74 ± 32.56</td>
<td>&lt;150.00</td>
</tr>
</tbody>
</table>

The treatment was carried out with increasing weekly doses; 100 mg (Week 1), 200 mg, 500 mg (Week 3), 1000 mg, 1500 mg and 2000 mg (Week 6). Values are means ± standard deviation.

Compounds cause astringency and a dry mouth and are very common in certain types of beverages such as red wine and tea (Camellia sinensis) (Bandyopadhyay et al., 2012). Because there is a high concentration of tannins in MIE extract (Carlini and Frochtingarten, 1988), it is likely that this sensation is related to the presence of this compound. In the case of the volunteers which had this reaction, there was also remission without the need for any measure or medical intervention.

Tests performed 21 days after the end of treatment confirmed the absence of toxicity, indicating that no late effect was triggered by MIE administration. These results complemented those obtained in preclinical studies (Tabach et al., 2017), which confirmed the absence of MIE toxicity. Furthermore, no significant changes were observed in biochemical or hematological profiles and psychomotor function throughout the treatment.

The fact that this medicinal plant has been accepted in the public health service, on the other hand, opens a new therapeutic option to the widespread use of proton pump inhibitors, which generally produce many adverse reactions, including fatal reactions. Thus, the results of this study confirm the broad popular use of the species, with no relate of adverse effects. This fact is very important in terms of public health. The MIE could, thus, represent a valuable alternative to treat common dyspepsia.

Acknowledgements

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Conflict of Interest

The authors have declared that there is no conflict of interest.

REFERENCES


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The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/uk/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:

1. **Replace (Ins) Tool** – for replacing text.
   - Strikes a line through text and opens up a text box where replacement text can be entered.
   - How to use it:
     - Highlight a word or sentence.
     - Click on the Replace (Ins) icon in the Annotations section.
     - Type the replacement text into the blue box that appears.

2. **Strikethrough (Del) Tool** – for deleting text.
   - Strikes a red line through text that is to be deleted.
   - How to use it:
     - Highlight a word or sentence.
     - Click on the Strikethrough (Del) icon in the Annotations section.

3. **Add note to text Tool** – for highlighting a section to be changed to bold or italic.
   - Highlights text in yellow and opens up a text box where comments can be entered.
   - How to use it:
     - Highlight the relevant section of text.
     - Click on the Add note to text icon in the Annotations section.
     - Type instruction on what should be changed regarding the text into the yellow box that appears.

4. **Add sticky note Tool** – for making notes at specific points in the text.
   - Marks a point in the proof where a comment needs to be highlighted.
   - How to use it:
     - Click on the Add sticky note icon in the Annotations section.
     - Click at the point in the proof where the comment should be inserted.
     - Type the comment into the yellow box that appears.
5. **Attach File Tool** – for inserting large amounts of text or replacement figures.

How to use it
- Click on the *Attach File* icon in the Annotations section.
- Click on the proof to where you’d like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

6. **Add stamp Tool** – for approving a proof if no corrections are required.

How to use it
- Click on the *Add stamp* icon in the Annotations section.
- Select the stamp you want to use. (The *Approved* stamp is usually available directly in the menu that appears).
- Click on the proof where you’d like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

7. **Drawing Markups** Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

How to use it
- Click on one of the shapes in the *Drawing Markups* section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.

For further information on how to annotate proofs, click on the *Help* menu to reveal a list of further options: